MICROBIOLOGICAL EXAMINATION OF FOODS

C.F.T.R.I.

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DIVISION OF MEAT & FISH TECHNOLOGY
Central Food Technological Research Institute
MYSORE.

Recommended Methods for the Microbiological Examination of Foods

The Subcommittee on Methods for the Microbiological Examination of Foods has prepared this report. It has been reviewed by the Coordinating Committee on Laboratory Methods and recommended for publication. Publication has been authorized by the Committee on Evaluation and Standards of the American Public Health Association.

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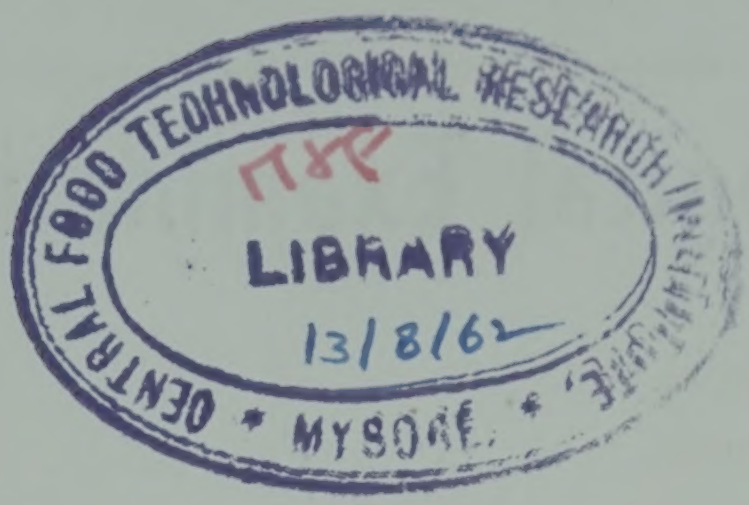

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PREFACE

One of the functions of the Coordinating Committee on Laboratory Methods of the American Public Health Association is to assemble from various sources the principal methods for performing laboratory tests in numerous public health laboratory fields. This is usually done by a subcommittee headed by a person with wide experience in the particular field. The Subcommittee on Methods for the Microbiological Examination of Foods, which prepared this volume, serves under the Coordinating Committee on Laboratory Methods.

The methods given in this, the first edition of "Recommended Methods for the Microbiological Examination of Foods," have been originated, developed, or assembled by Harry E. Goresline, Ph.D., and the members of his committee. Many of the original methods were developed by committees of the Food and Nutrition Section.

For several years laboratory workers interested in food microbiology have realized the need for a manual or publication such as this. These methods are not designated as "Standard Methods." Our committee realizes full well that there are other methods of possibly equal value. It is the hope of the committee that interested persons will submit or recommend improved, modified, or new methods that may be included in a second edition. Dr. Goresline and his committee should be commended for assembling in one volume a set of methods covering practically the entire field in food microbiology.

This volume represents a tremendous amount of work and effort. As is usual much of this was borne by the chairman of the committee. The chairman and members of the Coordinating Committee on Laboratory Methods wish to express their thanks and appreciation to Dr. Goresline for his diligent endeavor and for the hours of work he has given in the preparation of this first edition. We also wish to extend our thanks to the other members of Dr. Goresline's committee who have assisted him and also to the many others who have devoted their time and energy to this publication. It is our hope that this volume will fill a need and that it will prove useful to the many food microbiologists and others interested.

During the early preparation of this volume, the Coordinating Committee on Laboratory Methods was a subcommittee of the Committee on Research and Standards, first under the chairmanship of William McD. Hammon, M.D., and later of Malcolm H. Merrill, M.D. To Dr. Hammon and Dr. Merrill, and to their committees, we express our thanks.

For the Coordinating Committee on Laboratory Methods, American Public Health Association, June, 1958.

FERDINAND R. HASSLER, M.D., M.P.H., Chairman

CHAPTER I

GENERAL DIRECTIONS

SAMPLING

Whenever possible, submit samples of product to the laboratory in original unopened containers. This will prevent possible contamination and reveal the true condition of the product as prepared and offered to the public.

If products are in bulk, or in containers of a size making it impracticable to transport to the laboratory, transfer a representative sample to sterile containers under aseptic conditions. Since it is impracticable to sterilize certain sampling devices in the field, it is best to sterilize such devices in the laboratory and transport them in sterile carrying cases. Where drills, triers, agitators, etc., are used in the field it is often necessary to sterilize between samplings. Where this is necessary, special sterilizing equipment should be provided, as facilities in stores, warehouses, and food plants are seldom adequate for that purpose.

Where the food product is composed of several parts or components, such as a cream pie with meringue topping, turkey and dressing, etc., it is often desirable to determine the bacterial population or contamination of each component, or to determine the possible point source of an infection. In such cases the components should be sampled separately in such a way as to eliminate as much chance of cross-contamination as possible. In the case of a cream pie, take a sample of the topping without disturbing the filling and then with a sterile implement cut away the surface of the filling with a minimum chance of contamination from the surface and the topping. Only in this way can the exact position of the contamination in the product be determined. This could become important if the components were processed separately and in such a manner as to give rise to large numbers of microorganisms in specific portions and not in others.

Aseptic precautions should be taken at all times and in particular in opening containers to obtain samples.

SAMPLE CONTAINERS

Provide clean, dry, leakproof, wide-mouth, sterilized containers of a size suitable to hold samples of the particular product under examination. Use glass-stopper or screw-cap bottles, clamp or screw-type fruit jars, or other containers that are leakproof. For dry material, sterile metal boxes, cans, etc., with suitable closures, may be used.

TRANSPORTING OF SAMPLES

Transport samples to the laboratory as rapidly as possible, being sure to maintain the original conditions under which the material was held. If product is canned or in a dry condition no particular precautions are necessary, but if product is under refrigeration or is frozen, samples should be transported rapidly in a protective case so they will arrive at the laboratory in an unchanged condition. Frozen samples should be kept solidly frozen at all times. Thawed samples of frozen products should be discarded as results on such samples will have doubtful meaning.

Frozen samples should be collected in prechilled containers and placed in the freezer for sufficient time to become thoroughly frozen. Refrigerated samples should be transported in wet ice if available, otherwise in well insulated chilled containers. Samples of this class of product should not be frozen since destruction of certain microorganisms will occur.

PREPARING SAMPLES FOR EXAMINATION

Avoid all contamination when opening containers or sample bottles. Cans, jars, and other sealed containers should be washed, dried, and the area of opening sterilized by some suitable agent such as alcohol which is removed by burning if the package will not be harmed and under conditions where there is no hazard of fire or explosion. Paper cartons, cellophane packages, foil wraps, and similar packaging material should be wiped with a cloth or sponge dipped in a sterilizing agent to remove surface contamination and dust, and special attention given to the area of opening.

A truly representative sample should be taken. Agitate liquid or free-flowing materials until the contents are homogeneous. If the container

is too large to handle conveniently, sample by means of sampling tubes or triers, making sure that all parts of the package are represented in the sample. If material is in pieces, or a part must be cut or broken away, obtain material from several locations in order to represent the true condition of the package. Unwieldy material should be placed in a large sterile container and broken into smaller pieces by suitable means under aseptic conditions and a sample then transferred to a sterile container.

All equipment, pipettes, dilution bottles, Petri dishes, hot-air ovens, autoclaves, thermometers, incubators, water, and bacteriological media ingredients should meet the specifications of the APHA "Standard Methods for the Examination of Dairy Products." [1]

TEMPERATURE OF INCUBATION

Many different temperatures of incubation have been presented in the literature. To comply with all of the suggested temperatures would require a battery of incubators far beyond the capacity or budget of the average laboratory. Therefore, an attempt has been made to select a minimum of temperatures of incubation and those believed conducive to the best results from the type of food under test and for the specific types of organisms under consideration. Where intermediate or "odd" temperatures are given it is for a specific test which depends upon temperature regulation within a specific range.

The recommended temperatures of incubation set forth in this volume for most food products are 32° C, 37° C, and 55° C. For the growth of certain types of microorganisms 37° C is believed necessary as an incubation temperature, but for most types of organisms causing spoilage and deterioration of fresh or processed food products an incubation temperature of 32° C will give better results in the laboratory. In most cases the total plate count of food decreases as the temperature of incubation is raised above 32° C. If it is not possible to maintain the suggested temperatures of incubation the actual temperature should be stated in reporting results as there is likely to be a considerable difference in count at the different temperatures.

In the case of meat products held under refrigeration a temperature of 21° C is recommended for the growth of cold tolerant organisms and for the growth of yeasts and molds found in certain products.

In the chapter on Sanitation Indexes a temperature of 35° C has been given in order to comply with directions presented in "Standard Methods for the Examination of Water, Sewage, and Industrial Wastes." [2]

REPORTING RESULTS

Report results as numbers of bacteria, or other microorganisms or numbers of colonies of microorganisms, per gm or per ml, indicating type of material, temperature, and period of incubation. When liquid media technics are used for enumeration report as most probable numbers of organisms.

DIFFERENTIATION OF TYPE OF ORGANISMS

In most cases the use of differential media is sufficient for identification of types of organisms involved. Further differentiation may be made by observation under the microscope. When it is necessary to be more specific than the above methods it is recommended that the worker consult "Bergey's Manual of Determinative Bacteriology" [3] and "The Manual of Methods for Pure Culture Study of Bacteria." [4]

USE OF TEXT

Since special consideration must often be given to different types of food products, due to their form, acidity, temperature requirements, or public health significance, the field of food products has been arranged in groups which are similar, or can be assayed by the same general procedures and methods.

Foods of great similarity have been grouped into chapters and the methods presented designed for use with items of similar type, origin, form, or use. Cross-referencing between chapters has been carried out to some degree but in most cases specific methods are presented in entirety within the chapter in order to prevent confusion and to simplify the use of the material.

Abbreviations have been used for many common and easily recognized terms in the interest of space saving in the text. Examples are: min for minute, hr for hour, ml for milliliter, cm for centimeter, cc for cubic centimeter, sq cm for square centimeter, 1° F for 1 degree fahrenheit, gm for gram, *M* for molar, *N* for normal. It will be noted also that periods are not used after abbreviations.

Citation of literature in the text is made by a raised number, thus,[2] and the references corresponding to the numbers are presented at the

end of each chapter, thus keeping related material intact. References presented under Culture Media are in the form of similar raised numbers and the reference is given at the bottom of each medium write-up.

References to Culture Media, Reagents and Indicators, and Stains are set forth in the text as numbers set in parentheses with the subject of the citation being found in the Appendixes. Since most of the references are for Culture Media, the prefix letter "A" has been deleted. References to Appendix B and Appendix C use the respective prefix letters. Thus, (10) refers to Culture Medium Number 10 to be found in Appendix A, while (B2) refers to stain number 2 to be found in Appendix B.

INTERPRETATION OF RESULTS

In order that the worker may have some guidance in interpreting his results a section setting forth some experiences of other workers has been included in each chapter. It is to be understood that numbers given or interpretations made are in no way to be taken as a suggested standard. They are presented merely as guidelines to assist the laboratory worker in formulating opinions as to what his results may mean in terms of the product being examined. Good judgment must always be used in interpreting results. Particular conditions prevailing or the purpose for which the microbiological examination is being made may completely change the picture from that of a "normal" sample with "expected" low counts. Temperatures of incubation should be taken into consideration in evaluating results. When anything more than the reporting of counts on a given sample is required the laboratory worker should obtain a complete history of the sample before rendering an interpretation as to the meaning of the results.

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1. Standard Methods for the Examination of Dairy Products (10th ed.). New York: American Public Health Association, 1953.
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3. Breed, R. S.; Murray, E. S. D.; and Hitchens, A. P. Bergey's Manual of Determinative Bacteriology (6th ed.). Baltimore, Md.: Williams and Wilkins, 1948.
4. Manual of Methods for Pure Culture Study of Bacteria. Baltimore, Md.: Society of American Bacteriologists (Committee on Bacteriological Technique), 1932.

CHAPTER II

BOTTLED SOFT DRINKS

The need for microbiological examination in this field can be found in the public health aspects of the production, distribution, and widespread use of beverage products, and the need for the determination of the causes and prevention of spoilage.

The bottled soft drink beverage industry consists of approximately 5,400 plants, virtually all of which are locally owned and operated in most of the moderate or good sized cities, and which have a factory output equivalent to about 15 billion half-pint portions per year. The size of the plant and output depends upon the municipality and the area being served.

Of these beverages, most of which are carbonated, the commonly recognized flavors are the ginger ale, kola, citrus flavored, and herbal types such as root beers, etc. Most of the beverages are prepared from extracts or infusions of various roots, berries, barks, or herbs, or may be made from the juice and pulp of various fruits. In some of the flavors essential oils manufactured by various methods are employed, and in a few cases synthetic flavors are used. Substantially all of the beverages are acidulated with edible acids such as citric, phosphoric, tartaric, or lactic. They contain sugar which imparts to them much of their flavor and their caloric value, although some "dietetic" varieties may contain a noncaloric sweetener. Caramel and certified food colors are used to enhance the appearance of the product.

There is no evidence that carbonated beverages serve as a means for transmitting pathogenic organisms. However, as thirst quenchers, they should be free of undesirable organisms in order to maintain a status as an adjunct to the diet. Since the finished product does not undergo a sterilizing process examination of the finished product will be of advantage, not only for public health control purposes, but also for the routine maintenance of plant sanitation. In the same way, methods of examination of ingredients will serve as indicators of contamination in the plant and aid in the elimination of any undesired microorganisms showing up in the finished product.

The process of beverage preparation may be divided into 3 parts.

namely: (a) the washing of the returnable glass bottle, one trip bottles (nonreturnable), or cans; (b) the preparation of the flavoring sirups employed, together with water processing; and (c) the filling and hermetical closing of the container. Recently the sealed can has been added to the line of soft drinks but now claims only 1 to 2 per cent of the pack.

The majority of glass bottles employed are used for repeated refillings. The normal procedure is to wash them in a caustic soda containing solution, where, by the combination of causticity, temperature, and immersion time, the foreign materials are removed and the bacterial content is reduced to such a low level as to constitute commercial sterilization. One trip bottles or cans require less drastic washing. The actual checking of the effectiveness of the bottle washing procedure is a bacteriological control problem.

METHODS OF EXAMINATION

I. Water

All water used in direct preparation of the product and for cooling purposes should meet the requirements for drinking water of the U. S. Public Health Service.[1]

Sample—The sample should be at least 8 fl oz, collected in a sterile bottle, with either a glass or other impervious closure. Collect water entering the plant, after plant processing (filtering, chemical treatment, etc.), and as delivered at the filling machine head. If another source of water is used for general plant service or bottle rinsing, it should be similarly sampled.

Bacteriological Method—Use the methods prescribed by the APHA in "Standard Methods for the Examination of Water, Sewage, and Industrial Wastes," [2] making examinations for total bacterial count and coliforms.*

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* In employing the lactose fermentation tubes on finished beverage difficulty may be experienced by the acids in the beverage depressing the pH too low in the media; this can be buffered with 5 gm of K_2HPO_4 per liter of lactose broth. Because of the sugar present in some of the beverages, evidence of gas in the fermentation tubes may be due to yeast growth and not coliforms; therefore, any evidence of gas must be confirmed by transplanting into differential media for verification before reporting.

II. Sugar

Sugar used should be relatively free of potential spoilage microorganisms. Spoilage factors in beverages are materially affected by the quality of the sugar, as well as its packaging and handling up to the time of use.

Sample—Obtain at least ½ lb of sugar, representative of the lot being used, taken in a sterile, wide-mouthed jar, using a sterilized spoon or cup and taking precautions to prevent secondary contamination.

Microbiological Method—Weigh 20 gm of sugar aseptically into a sterile container and dilute to 100 ml with sterile water. (Each 5 ml of the resulting sirup will contain 1 gm of sugar.)

Pipette 1 ml portions into 2 sets of 5 Petri plates each. Pour the plates with nutrient agar (45) for making total counts, and with potato dextrose agar, acidified (51), or malt agar (36) for yeasts and molds. Incubate at 32° C for 3 days for total count and count at 3 and 5 days for yeasts and molds.*

Average the count of the 5 plates and report as numbers of mesophilic colonies, and as yeast and mold colonies per 10 gm of sugar.

III. Sirup (Liquid Sugar)

Sirups delivered to the plant, as well as sirups prepared in the plant, should be relatively free of microorganisms which may promote spoilage.

Sample—Take at least 100 ml of sirup collected aseptically in a sterile jar or bottle and representative of the lot. Where sirup undergoes any processing or mixing with other ingredients take samples before and after each step.

Microbiological Method—Since the concentration of the sirup may vary, the portion measured should result in a 20 per cent sugar solution when diluted with sterile water to 100 ml.

Follow the recommended procedure under "II—Sugar."

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* Observing plates at both 3 and 5 days is advisable for reliable yeast count as mold growth may cover and obscure the surface before the end of incubation.

IV. Flavoring and Coloring Ingredients

In addition to sugar, all solutions of flavoring, acidulents, colors, etc., should be relatively free of spoilage microorganisms.

Sample—Sample at least 50 ml of the particular stock solution taken aseptically in a sterile jar or bottle. If in dry form make up a stock solution for sampling.

Microbiological Method—Unless there is evidence of extremely high contamination as indicated by odor or change in appearance, the following routine should be used: *

Pipette 1-ml and 0.1-ml portions of undiluted sample into 2 sets of 2 plates each.† Pour plates of 1 set with nutrient agar (45) for total count, and the other with potato dextrose agar, acidified (51) or malt agar (36) for yeasts and molds. Incubate at 32° C and count after 3 days for total count, and count at 3 and 5 days for yeasts and molds.

Average the counts of the plates and report as numbers of mesophilic colonies and as yeast and mold colonies per ml of original solution or per gm of dry material.

V. Washed Bottles

The washed bottles before filling should be free of all harmful or spoilage types of organisms and contribute few if any microorganisms to the product being filled into them.

Sample—Take at least 5 bottles at random from the discharge end of the bottle washer over a 1-min period. If more extensive study is desired, take at least 1 bottle from each discharge row of machine during a 5-min period. If the bottles are not to be analyzed immediately, close with sterile stoppers, store at 4° C (40° F) and analyze within 4 hr after collection.

Microbiological Method—a. For routine examination of bottles which permit observation of the insides (i.e., transparent color and no exten-

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* Where solutions are used which contain benzoate of soda or alcohol, attention must be given to have sufficient media or dilution to reduce the preservative effect to negligible proportions.

† With high infection, plate 1:100 and 1:1,000 ml, using water dilution blanks.

sive decoration), pour 10 ml of nutrient agar (45) into the bottle and roll it thoroughly to cover all portions of the inside. When cool allow the bottle to remain on its side for incubation at 32° C and count at 3 and 5 days. Colonies which grow will likely indicate yeast, mold, or bacterial contamination and lack of proper cleansing.

b. For more extensive examination, pour 10 ml of sterile water plus a small amount of sterile quartz sand into the bottle and swirl the liquid and sand thoroughly over the inside surfaces. Pipette aliquot portions into 2 sets of Petri plates in duplicate, and pour 1 set with nutrient agar (45) and the other with potato dextrose agar, acidified (51) or malt agar (36). Incubate at 32° C and after 3 days make total count and at 3 and 5 days make yeast and mold count. Report the colonies counted as bacteria, yeasts and molds per bottle. If coliform index is desired see Chapter XVI.

c. For containers that are not transparent culturing must be done by method *b*. This should be used with metal cans whether they be flat-top design, without the top end double-seamed, or the cone top type intended for crown closure. The cans should receive 10 ml of sterile water plus a small amount of sterile quartz sand, given a swirling motion over the inside surfaces and followed by pipetting of aliquot portions into the desired media as indicated under *b*. The same reporting of counts should apply.

VI. Closures

The inner sealing surface of the closures or can ends which comes in contact with the contents of the container should always be free from organisms which might in any way affect the product.

Sample—Select at least 10 crown closures or can ends at random from each unopened manufacturer's package. If they are selected from a hopper or open bin, a representative sample of from 10 to 100 pieces should be taken, depending upon the number present. Select closures with sterile tongs and place in a sterile jar taking care not to contact the inner surfaces.

Microbiological Method—Remove the crowns or can ends from the jar with sterilized tongs. Flame very lightly on the back side of the shell, taking care not to expose the inner surface to the flame, or wipe the back with 70 per cent alcohol. Place crowns or can ends bottom

side up in sterile Petri plates and pour agar with a pipette on the liner surface sufficient to fill or cover the upended closure. Use nutrient agar (45) for total count and potato dextrose agar, acidified (51) or malt agar (36) for yeast and mold count.

Incubate at 32° C and after 3 days make total count and count at 3 and 5 days for yeast and mold count. Report as colonies of mesophilic bacteria, yeasts, and molds per piece.

An alternate method is to drop 5 of the crown closures or can ends into 25 ml of sterile water containing sterile quartz sand and agitate vigorously. Transfer 5-ml aliquot portions of the water into 2 sets of duplicate Petri plates and pour with nutrient agar (45) and potato dextrose agar, acidified (51) or malt agar (36). Incubate at 32° C for 3 days and make total count. Incubate yeast and mold plates at 32° C and make counts at 3 and 5 days. Report results as colonies of mesophilic bacteria yeasts or molds per piece.

VII. Finished Beverage

The finished beverage should meet the requirement for drinking water according to the Public Health Service Drinking Water Standards.[1] In addition, they should be relatively free of organisms potentially responsible for spoilage.

Sample—Select at least 2 representative bottles or cans of product taken in a 3-hr period of plant operation, or from stock at point of sale.

Microbiological Method—Open the bottle aseptically and flame the top lightly. Flat-top metal cans should be washed, the end wiped with alcohol, flamed and pierced with a sterile opener. Using a 10-ml pipette, having 0.1 ml markings, withdraw a sample of the beverage. (If a smaller pipette is used, difficulty will be experienced with gassing of the beverage.)

Pipette 1-ml portions and 0.1-ml portions into 2 sets of 2 plates each. Pour 1 set of plates with nutrient agar (45) for total count and the other with potato dextrose agar, acidified (51) or malt agar (36) for yeasts and molds. Incubate at 32° C. Make the total count at 3 days and the yeast and mold count at 3 and 5 days.

Average the colony count of the plates and report as colonies of mesophilic bacteria, and as yeasts and molds per ml of beverage.

VIII. Analysis of Beverage Cooler Water

Beverages are often cooled at the point of sale and consumption by immersion in water chilled either by mechanical refrigeration or added ice. The transfer of any organisms in the cooling water to the consumer at the time of consumption of the beverage may, therefore, be of sanitary concern.[4] The total count of organisms in the water may be determined by plating aliquot portions as described in "Standard Methods for the Examination of Water, Sewage, and Industrial Wastes." [2]

INTERPRETATION OF RESULTS

Bottled beverages are consumed as thirst quenchers. As such they should meet or be better than the requirements for drinking water as regards the various types of organisms. Whenever the finished beverages do not meet the requirements for drinking water according to Public Health Service Drinking Water Standards,[1] there is definite indication of unsatisfactory raw materials or very faulty processing methods. In most cases the primary source and carrier for coliform organisms is the water supply employed, but in rare instances there have been found human contamination sources within the processing plant which lead to excessively high counts of these organisms.

The bottles employed as the package for these beverages are commonly washed in strongly alkaline solutions, usually specified as to strength, temperature, and time of exposure by the various states. In most cases the bottles are those returned by customers but unless the bottle is grossly contaminated, by massive amounts of residual foreign materials, it is not the carrier or source of contamination in the beverage. One trip bottles or cans must be adequately washed to remove dust, etc., and should not be a source of such organisms. The crowns or can ends used on the containers have not been indicated as a common source of this type of infection.

The presence of yeasts, molds, or other closely related organisms is normally found as an irregularity in raw materials or processing methods within the bottling plant. Sugars (or liquid sugars) which are not "bottlers sugars" may be a very serious potential source of infection in sirups and in the ultimate flavoring materials used in the manufacture of the beverage. If the laboratory worker is interested in suggested standards for such products see reference 3. Flavoring and acid solutions

which do not contain a preservative or have not been pasteurized can develop growths which are ultimately transferred into the product. Counts of approximately less than 2 yeasts per ml of acidified beverage may not develop product deterioration but are considered marginal in character. The inhibitory characteristics of carbon dioxide and acid may repress growth to such an extent that yeasts do not reach the numbers necessary for flavor change or production of sediment. Higher numbers of organisms, however, invariably tend to form sediments, discoloration, and definite flavor change in the product, and are therefore a mark of low quality. Their elimination is principally a matter of proper specification and control of raw materials as well as the use of modern equipment under carefully controlled, sanitary manufacturing methods.

Occasionally, massive growths of molds may appear as a gelatinous to webby mass within the products or as a sediment. Normally this is an indication of gross contamination and faulty manufacture of the flavoring sirups, or quite often, improperly washed bottles. The particular characteristics of the mold causing such growths are quite difficult to identify from direct microscope examination, and should always be cultured for true morphological detail.

With modern, well designed, high speed, efficient, and sanitary machinery for the preparation of the flavoring sirups, for the purification and control of the water supply used, and for the washing and sanitizing of the containers used, the organism counts found in these products are normally exceedingly low, and in most instances approach zero.

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CHAPTER III

BRINED, SALTED, AND PICKLED VEGETABLE PRODUCTS

The methods described here * should prove useful to those concerned with the examination of certain types of manufactured pickles, particularly those products undergoing spoilage as the result of microbial activity. The methods also should prove helpful to research workers interested in conducting studies on predominating microbial changes occurring in certain brined and salted vegetables during natural fermentation and curing.

CLASSIFICATION

Brined, salted, and pickled vegetables are divided into the following groups:

I. Cucumber Pickles and Similar Pickle Products

A. *Salt Stock for Cured Pickle Products*

1. Cucumbers (and onions, peppers, tomatoes, cauliflower, melon rinds, etc.)
2. Genuine dill pickles (from cucumbers or tomatoes)

B. *Finished Pickle Products from Brine-Cured Stock*

1. Sweets
2. Sours
3. Mixed
4. Relishes
5. Artificial or processed dills

C. *Types of Pasteurized Pickles (Not Brine-Cured)*

1. Dills (sliced or whole)
2. Sweets (sliced or whole)
3. Relishes (mixed vegetables)
4. Vegetables other than cucumbers (onions, peppers, tomatoes, etc.)

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* In large part revised from the original publication by Etchells, J. L., and Jones, I. D. Procedure for Bacteriological Examination of Brined, Salted and Pickled Vegetables and Vegetable Products. A.J.P.H. 36:1112–1123 (Oct.), 1946.

II. Brined and Salted Vegetables for Nonpickle Use

A. *Brined*

1. Okra (whole)
2. Celery (whole)
3. Sweet pepper hulls

B. *Dry-Salted*

1. Corn
2. Lima beans
3. Peas
4. Snap beans
5. Okra (cut)
6. Celery (cut)

GENERAL PROCEDURE

I. Collection, Storage, and Preparation of Brine Samples

Brine or pickle liquor covering vegetable material is required for examination. The size of container to be sampled may range from a small jar of pickles to a 1,000 bushel vat of fermenting salt stock. Brine samples from large containers, such as vats and barrels, should be taken for bacteriological analysis as follows:

A suitable length of 3/16″ stainless steel tubing (sealed at one end with lead or solder and perforated with several 1/16″ holes for a distance of 6–8″ from the sealed end) is inserted, through an opening in the false head, into the brine toward the center of the vegetable material. Withdraw brine through a previously attached piece of rubber tubing into a 12-oz juice bottle. The receiving bottle is fitted with a 2-hole rubber stopper and 2 short lengths of glass tubing—one for the rubber tubing leading from the stainless steel sampling tube, and the other for a suction bulb to start siphoning action. The length of steel sampling tube is governed by the depth of the container to be sampled.

Withdraw approximately 24 oz of brine before taking the final sample (about 10 ml) in a sterile test tube. If microbial changes during the fermentation are to be followed, start sampling at the time material is salted or brined and continue at regular intervals of 1 to 2 days during active fermentation.

For tightly-headed barrels, such as used for genuine dills and salted vegetables for nonpickle use, take sample through the top or side bung.

For smaller containers, such as jars or cans of pickle products, shake thoroughly and take from center of the material by use of a sterile pipette. Wash the tops of metal cans with alcohol, flame, and puncture. A beer can opener is useful for puncturing metal tops. If the containers show evidence of gas pressure, release gas carefully by puncturing top with a flamed ice pick.

II. Storage of Samples

Brine samples from actively fermenting material should be examined as promptly as possible after collection to prevent changes in the microbial flora present. The same is true for samples of packaged pickle products. If it is necessary to ship or store samples this should be done under the best of refrigerated conditions and the elapsed time from collection to examination should preferably not exceed 12 to 24 hr. Samples collected in sterile 16 x 150 mm test tubes, fitted with plastic screw caps having pulp-backed vinylite and teflon liners, are useful where shipment is required.

Brine samples can be preserved for subsequent chemical determinations by addition of sodium, 2, 4, 5 trichlorphenolate (Dow) to make a dilution of about 1:10,000 as described by Veldhuis.[1] Collect samples in standard 3- to 4-oz medicine bottles and add about 10 drops of a 10 per cent aqueous solution of the chemical. Shake well to distribute preservative. Caps having pulp-backed vinylite and teflon liners, or pulp-backed foil liners should be used, as those having cork or composition cork are not satisfactory for prolonged storage. If samples are to be tested for enzyme activity, use 10–15 drops of toluene as a preservative instead of sodium, 2, 4, 5, trichlorphenolate.

Samples preserved with the above chemicals are unfit for human consumption and *should be so marked*.

III. Preparation of the Sample

Make suitable dilutions of the pickle liquor or brine in the usual manner, except for obligate halophiles. For this group, make serial dilutions directly into the recommended liquid medium containing salt. If poured plates, using salt containing media are desired, the dilution blanks should contain approximately the same salt concentration as the brine sample. For actively fermenting brines no specific number of dilutions can be suggested; however, as a guide such brines may be

expected to contain the following populations per ml: acid-forming bacteria, 10:1,000 million; yeasts, 10:100 million; obligate halophiles, 10:1,000 million; coliforms, 1:10 million; salt-tolerant cocci, 1:10 million. The expected microbial populations in adequately pasteurized products are normally very low and composed of resistant spore-forming bacteria that remain dormant in the acid liquor. For such products, dilutions of 1:10 and 1:100 usually suffice. For improperly pasteurized products that are fermenting, the dilutions should cover the estimated range of population suggested for acid-formers and yeasts in fermenting brines.

IV. Microscopic Examination

Microscopic examination of brine samples for bacteria and yeasts is helpful at times, particularly when carried out in conjunction with plate count observations.

A. *Technic for Bacteria*

Make direct counts for bacteria according to the following procedures: Place 0.01-ml portions of brine or liquor on slides, by using a Breed [2] pipette, and spread evenly over a 1-sq cm area. Prepare and count according to the Wang [3] modification of the Breed [2] technic. Stain according to the Kopeloff and Cohen [4] modification of the Gram stain (B4). Report results as numbers of different morphological types of Gram-positive and Gram-negative bacterial cells per ml of brine.

B. *Technic for Yeasts*

The microscopic technic can be used to advantage for determining yeast populations in fermenting vegetable brines and various types of finished pickle products undergoing gaseous spoilage by these organisms. It provides a rapid and reliable method for detecting and following yeast activity, particularly where populations are in excess of 10,000–20,000 per ml of sample, and where yeast colonies are not required for isolation and study. Furthermore, the use of a vital stain permits differentiation of yeast population into viable and nonviable cells, and increases the value of the direct counting technic.

Yeast counts of viable cells of subsurface species in fermenting cucumber brines may average 4–5 times that obtained by the plating technic.

Clusters of viable cells no doubt are responsible for this difference as each clump of cells forms a single colony on a plate while the actual number of cells are recorded in the microscopic method.

It should be emphasized that neither microscopic nor the plate counting technics give a true picture of the populations of gas-forming, subsurface species of yeasts in fermenting brine or pickle samples obtained from containers also contaminated with film yeasts originating from luxuriant surface growth.

The procedure is essentially the method of Mills [5] as modified by Bell and Etchells [6, 7] for counting yeasts in high salt content brines and in high sugar content liquors:

Add 1 ml of brine or pickle liquor sample to 1 ml of 1:5,000 (0.02 per cent) erythrosin stain (B1).

Shake the sample stain mixture to obtain an even suspension.

Using a 3-mm diameter platinum loop transfer enough of the mixture to the area under the cover glass of an improved Neubauer double-ruled hemacytometer to fill chamber in one operation.

Allow cells to settle for approximately 5 min and count yeast cells, using a microscope equipped with a 4-mm objective and 15 x oculars.

Record cells stained pink as "dead yeast cells," and unstained cells as "live yeast cells."

The number of yeast cells per ml of brine or pickle liquor may be calculated thus:

$$\frac{\text{Number of yeast cells counted x dilution x 250,000}}{\text{Number of large squares counted}} = \text{numbers per ml.}$$

If only one side of the hemacytometer counting chamber is used (25 large squares) the lowest yeast count obtainable is 20,000 per ml, while if both sides are counted (50 large squares) a population as low as 10,000 per ml can be counted.

Report yeast count as (a) total yeast cells, (b) live yeast cells, and (c) dead yeast cells, per ml of sample.

V. Titratable Acidity and pH

Determinations of titratable acidity and pH of the samples are extremely useful in providing supplementary information to bacteriological analysis.

Determine titratable acidity on a 10-ml sample of the brine or liquor by diluting sample with 30–50 ml of distilled water, bring just to boiling, cool, and titrate with 0.1 *N* NaOH, using phenolphthalein as the indicator. Report values for brined samples in terms of gm of lactic acid per 100 ml of sample and for finished liquor samples in terms of gm of acetic acid per 100 ml of sample.

When a 10 ml sample is titrated the following may be used in making the calculations:

A. ml of 0.1*N* alkali used x 0.111 = gm of lactic acid per 100 ml.
B. ml of 0.1*N* alkali used x 0.074 = gm of acetic acid per 100 ml.

Where only a small amount of original sample is available, a 2-ml sample may be used for titration purposes, although such small samples are not recommended.

Make pH-determination with a glass electrode, after aeration of sample to remove dissolved carbon dioxide.

VI. Determination of Salt Content of Brine

Knowing the approximate salt content is often helpful in making the microbiological examination of brines. This can be obtained by a salometer using about 200 ml of brine. For small amounts of sample, or where a higher degree of accuracy is desired than that obtainable by the salometer, a chemical test for salt is required. The following method is recommended for use: Transfer 1 ml of sample to a flask and dilute with 15–20 ml of distilled water. Titrate with 0.171*N* silver nitrate solution (C4) (29.063 gm per liter) using 3–5 drops of 0.5 per cent dichlorofluorescein (C6) [8] as the indicator. Agitate to keep the precipitation broken up until a light salmon-pink color is developed. Report as gm of sodium chloride per 100 ml of sample.

When 1 ml of sample is titrated each ml of silver nitrate solution is equal to 1 gm of sodium chloride per 100 ml.

PROCEDURE ACCORDING TO TYPE OF PRODUCT

I. Cucumber Pickles and Similar Pickle Products

The three main classes of products under this heading are: salt-stock vegetables and genuine dills; finished or packaged pickle products made

from salt stock; and pasteurized pickle made from fresh stock. The cucumber is the principal vegetable involved, although substantial amounts of other vegetables, such as onions, peppers, cauliflower, and green tomatoes, may be used in mixed pickle, relishes, or as individual products.

A. *Salt-Stock Vegetables and Genuine Dills*

The determination of numbers and types of microorganisms should be carried out by use of the plating technic with differential media and with decimal dilutions in differential liquid media. Place decimal dilutions of samples in Petri plates, in duplicate, and pour with media with respect to the following:

1. *Total Count*—Use nutritive caseinate agar (46) and incubate for 3 days at 32° C. The same set of plates may be used for counting the lactic acid-forming bacteria and the salt-tolerant cocci.

2. *Lactic Acid-Forming Bacteria*—Use nutritive caseinate agar (46), incubate 3 days at 32° C, and count those colonies showing a zone of precipitated casein and a yellow halo around the colony. The V-8 medium (72) may be used for differentiation of these types of bacteria. Lactobacilli colonies appear green to black with a yellow halo.

3. *Salt-Tolerant Cocci*—Use nutritive caseinate agar (46) and incubate for 3 days at 32° C. Count colonies that are grayish white, entire, glistening and of moderate size, and similar colonies that are light orange to yellow in color. Subsurface colonies are lenticular to elliptical in shape. To effect identification when yeasts are present make stained preparation and examine under microscope.

4. *Coliform Types of Bacteria*—Use brilliant green lactose bile agar (12), violet red bile agar (73) or desoxycholate lactose agar (16). Incubate for 18–24 hr at 32° C.

5. *Yeasts and Molds*—Use dextrose agar, acidified, (17) and incubate 3–5 days at 32° C.

6. *Film Yeasts*—For an estimate, pick representative colonies from the yeast plates into tubes of dextose broth (18) containing 5 and 10 per cent salt. Incubate 3–5 days at 32° C, and observe for heavy surface film. Two salt concentrations are suggested for use because some species develop heavier films at the lower salt strength (5 per cent) whereas with other species the reverse is true.

7. *Obligate Halophiles*—Use tubes of liver broth plus salt (33). Prepare decimal dilutions, seal with melted petroleum jelly, and incubate 7 days at 32° C. Record positive tubes daily by noting the raising of the petroleum seal due to gas production, and absence of any distinctive odor.

8. *Butyric Acid-Forming Bacteria*—Neutralize the brine sample with an excess of sterile calcium carbonate. Heat a 50- to 100-ml sample in a water bath for

20 min at 80° C to kill vegetative cells. Prepare decimal dilutions and inoculate previously heated and cooled tubes of liver broth medium (32). Seal with melted petroleum jelly and incubate 7 days at 32° C. Record positive tubes daily as evidenced by production of gas and a strong butyric acid odor.

9. *Significance of Observations*—The acid fermentation resulting from active growth of lactic acid bacteria, is to be expected at brine concentrations below 15 per cent strength.[9] The acidity developed in the brine, in combination with the salt, results in preservation of salt stock and genuine dills.

Activity by coliform types of bacteria, obligate halophiles, and yeasts is associated with a gaseous fermentation. This may bring about a condition in salt-stock cucumbers and dill pickles known as "bloaters" or hollow cucumbers. Although these groups of organisms are extremely salt tolerant the coliform bacteria and halophiles are not usually found in brines having appreciable acidity. An exception may be found in cases of highly buffered material, such as dry-salted peas.

Luxuriant growth of film yeasts may occur at various salt concentrations and will result in loss of brine acidity. When certain molds accompany this scum growth, the vegetable material may become so soft as to be unusable. Heavy scum yeast and/or mold growth is the result of neglect in looking after brined material during the curing and storage period.

The significance of the presence of the salt-tolerant cocci and obligate halophiles is presented under Brined and Salted Vegetables for Non-pickle Use.

B. *Finished Pickle Products*

Fully cured salt-stock vegetables are made into various types of finished pickle products by a series of operations involving leaching out most of the salt, souring with vinegar, and then sweetening with sugar. In these finished products, preservation is dependent upon sufficient amounts of vinegar alone (for sour pickles) or a combination of vinegar and sugar (for sweet pickles). If amounts are inadequate, fermentation usually takes place, principally by 2 groups of organisms—lactic acid-forming bacteria and yeasts. Molds and film yeasts may also grow on the surface of the liquor chiefly as the result of faulty jar closure.

Liquor of the sample should be examined for (a) total number of microorganisms, (b) acid-forming bacteria, (c) yeasts and molds, and (d) film yeasts using methods set forth in I, A. Salt-Stock Vegetables.

In undisturbed containers the surface growth of molds and film yeasts may be obvious. Carefully remove the film after recording the extent of growth, since it will complicate the counts for acid formers and yeasts when the latter groups are present, if shaken up with the sample. Examination for coliform bacteria, salt-tolerant cocci, halophiles, and butyric acid bacteria is not normally required due to acidity of these products.

Significance of Observations—A total count of a few thousand organisms per ml is normally found in unspoiled pickle products. These counts are composed chiefly of resistant, aerobic spore-forming types that remain inactive in the acid medium of the pickle liquor. Active yeast fermentation in the product is usually characterized by vigorous gas production so that the liquor becomes highly charged with gas and possesses a stinging taste. Gas production may be sufficient to blow lids on jars having vacuum-type closures, to break jars having screw-type lids; or to burst sealed cans.[10] Also, whole pickles may become "bloaters" (hollow) due to the gaseous fermentation by yeasts and/or gas-producing types of acid-producing bacteria.[10]

The acid content of the liquor may be increased due to activity of acid-producing bacteria.

Extensive mold and film yeast growth usually result in a reduction in acidity of the liquor, and, in advanced stages, the vegetable may be softened due to such growth.

C. *Pasteurized Types of Pickle*

In general, pasteurization to an internal product temperature of 165° F, for 15 min, followed by prompt cooling, is required for pickle products that do not contain sufficient amounts of added vinegar and sugar to stop fermentation by certain organisms.[10] There are probably a dozen or more different types of cucumber pickle that fall into this classification, such as various types of fresh dills, fresh sliced cucumber pickle, and low-acid sweet pickle (from salt stock). Also, many noncucumber products are included: dilled tomatoes, sweet peppers, and fresh vegetable relishes that are prepared from uncured stock.

Spoilage occurs in these classes of products when they are improperly pasteurized, and is due chiefly to yeasts and/or acid-forming bacteria that survive faulty heat treatment. Molds and film yeasts are factors principally in cases of poor jar closure.

Examination of the liquor from the products of this class should be made as set fourth under I, B. Finished Pickle Products.

The significance of organisms found in the products is essentially the same as described under B. Finished Pickle Products. Since the acidity is often lower than in finished pickles, and the products may be made from fresh uncured vegetables, it is essential that spoilage types of organisms be detected, and that improper processing be recognized and corrected.

II. Salted and Brined Vegetables for Nonpickle Use

A. *Salted Vegetables*

Most of the vegetables, after blanching, are preserved according to the dry salt method,[11] using a ratio of 1:5 with respect to salt and vegetable weight, and stored in tightly-headed wooden casks, preferably at refrigerator temperature within the range of 1.7° to 4.4° C (35–40° F). Vegetables treated in this manner are generally green peas, corn, snap beans, cut okra, and cut celery. These salted vegetables are used in preparation of soups, mixed vegetables, and strained vegetable products.

B. *Brined Vegetables*

Products such as whole okra, whole celery, and sweet red peppers are usually brined at about 20 per cent salt concentration rather than by the dry-salt method. They are used in the same manner as the above salted vegetables.

Microbiological Examination—Brine samples from both A and B types of these vegetables should be examined for: total count, lactic acid-forming bacteria, salt-tolerant cocci, coliform bacteria, yeasts and molds, film yeasts, obligate halophiles and butyric acid-forming bacteria by the directions set forth in I, A Salt-Stock Vegetables and Genuine Dills.

1. *Significance of Observations*—In these products, gaseous fermentation is associated with active development of coliform bacteria, yeasts, and obligate halophiles, all of which can tolerate the high salt concentrations (15 per cent and above) normally employed for preservation. One or more of the above groups may be present. Gas pressure may be sufficient to burst the barrels. The flavor and appearance of the material may also be altered by growth of the above groups.

Numbers of salt-tolerant cocci may be found over an extended period in brines, particularly in those containing no appreciable amount of developed acidity.

Table 1—Guide to the Bacteriological Examination of Salted, Brined, and Pickled Vegetable Products

Microbial Group Involved	Culture Medium	Classes of Products in Which Microbial Group Is Likely to Be Present *	Remarks Concerning Microbial Groups
Total count	Nutritive caseinate agar (46)	All classes of products IA and B, and IIA and B	For determination of general microbial populations. In pasteurized products helps to indicate the effectiveness of the treatment.
Acid-forming bacteria	Nutritive caseinate agar (46) V-8 Medium (72)	IA: Fermenting salt-stock vegetables and genuine dills. IB: Finished pickle products. IC: Pasteurized pickle products.	Acid fermentation. Salt-tolerant up to 15 per cent; not likely to be found in brined and salted vegetables above this concentration. (IIA and B)
Salt-tolerant cocci	Nutritive caseinate agar (46)	II: Brined and salted vegetables for nonpickle use; also, other high salt vegetables without appreciable acidity.	No outstanding characteristics of fermentation reported. Group salt-tolerant but sensitive to acid. Can grow at refrigerator temperature (1.7° C) at approximately 10 per cent salt.
Coliform bacteria	Brilliant green lactose bile agar (12), violet red bile agar (73), or desoxycholate agar (16)	IA: Fermenting salt-stock vegetables and genuine dills. IIA and B: Brined and salted vegetables for nonpickle use.	Gaseous fermentation. Group salt-tolerant but not acid-tolerant. Most likely absent from finished pickles due to acid content; same is true for brines when appreciable acid is present.

* Refer to outline for more detailed classification of products listed under IA, B, and C, and IIA and B.

Table 1—(Continued)

Microbial Group Involved	Culture Medium	Classes of Products in Which Microbial Group Is Likely to Be Present *	Remarks Concerning Microbial Groups
Obligate halophiles	Liver broth plus salt (33)	IIA and B: Brined and salted vegetables for nonpickle use. Also, in other vegetable brines at high salt concentration.	Gaseous fermentation. Group requires 5–15 per cent salt in culture medium and reduced oxygen tension. Sensitive to acid. General information on behavior not well known.
Fermentative yeasts, film yeasts and molds	Dextrose agar (acidified) (17) Dextrose broth plus salt † (19)	All classes of products (IA, B, and C, and IIA, B), for yeasts. Molds and film yeasts on liquid surface of products exposed to air and sheltered from sunlight.	Yeasts: gaseous fermentation; acid- and salt-tolerant. Molds and film yeasts: acid- and salt-tolerant; both groups utilize acid of products and require free oxygen for growth.
Butyric acid group	Liver broth medium without salt (32)	Uncommon in brined and salted vegetables; examination should be made if malodorous fermentation is detected.	Causes malodorous, gaseous fermentation. Not particularly acid- or salt-tolerant. Active fermentations rare in properly brined or salted vegetables.

* Refer to outline for more detailed classification of products listed under IA, B, and C, and IIA and B.
† For culturing film forming yeasts in general.

These organisms are extremely salt-tolerant but not acid-tolerant. Their fermentation is not gaseous in nature and no outstanding change in the product has been attributed to their presence, although small amounts of brine acidity may be produced under conditions providing reduced oxygen tension. When numerous colonies showing a decided acid reaction are found on the plates, they should be carefully examined, as it is likely that they will not be acid-producing bacteria of the lactic group, as might first be suspected, but rather acid-producing cocci. This is particularly true in cases where the brine concentration is above 15 per cent salt.

Growth of molds and film yeasts is likely to be a factor where there is air above the brine surface in the container. Casks should be kept filled with brine at all times, irrespective of storage temperature. Unrestricted growth by molds may soften the texture of vegetable material so it is unusable. Heavy scum growth is undesirable, principally from the flavor standpoint.

As mentioned above, refrigerated storage (about 1.7° C) of these brined and salted products is preferred. Under such conditions, and at salt concentrations of 5 per cent and above, microbial activity of the various groups may be greatly restricted. However, at salt concentrations of approximately 10 per cent strength the cocci may grow rapidly at about 1.7° C (35° F).

SUMMARY OF PROCEDURE

A summarization of the bacteriological methods described herein is presented in Table 1. This information is suggested for use as a guide in the examination of certain brined, salted, and pickled vegetables and vegetable products.

DISCUSSION ON USE OF CULTURE MEDIA AND TYPES OF MICROORGANISMS

I. Nutritive Caseinate Agar (46)

This medium has been found very useful because several types of bacteria can be detected and enumerated on the same Petri plate, thus saving time, effort and glassware. It can be used for enumeration of total bacterial count, acid-forming bacteria and salt-tolerant cocci, and for determining population trends of acid-producing bacteria in dill pickles,[12] salt-stock,[9, 13] improperly pasteurized fresh cucumber pickle,[14] and in the storage of salted and brined vegetables.[15, 16]

Since this medium contains less agar than usual solid media, care should be exercised in amount poured per plate. Not over 15 ml should

be used to insure solidification and prevent dropping of agar when plates are inverted. During hot weather plates should be cooled prior to inversion and incubation.

In the presence of bromcresol purple, acid-forming bacterial colonies show a zone of precipitated casein and a yellow halo. The degree of casein precipitation and color change may vary with the activity and type of acid former.

Surface growth is usually poor. Subsurface colonies are generally elliptical in shape and range in size from 0.5 to 2.5 mm.

Yeasts, other than lactose fermenters, do not grow well on this medium and tend to give a slightly alkaline reaction. In cases of doubt, stained preparations should be made. Occasionally a high percentage of tiny (0.1 mm or less) acid-forming colonies is found in certain vegetable fermentations. Growth of these poor lactose-fermenters is often greatly enhanced by addition of 0.1 per cent dextrose to the medium. The additional dextrose will likewise enhance the growth of yeasts if present in the sample. For this reason careful examination for yeasts should always be made when the dextrose supplement is added to nutritive caseinate agar.

While nutritive caseinate agar is not considered a differential medium for salt-tolerant coccus forms, the numbers of these organisms in brines of high salt concentration may be estimated. They are indicated by two predominating types of colonies: one grayish white, entire, glistening and of moderate size, and a similar colony that is yellow to light orange in color. In high salt content nonacid brines, these organisms are the principal types found on this medium. Due to sensitivity to acid they are not usually found in active fermentations of the acid type. Deep subsurface colonies may give an acid reaction but upon prolonged incubation become alkaline. In highly buffered salted vegetables, bordering on the range of salt tolerance for acid-forming bacteria, care should be exercised that deep colonies of cocci are not recorded as true acid-producing bacteria of the lactic group.

II. V-8 Medium (72)

For determining numbers of lactobacilli, Fabian, et al.,[17] have shown this medium to give good results. The colonies are green to black with a yellow halo, and develop to a large size in the presence of the lactose. The bromcresol green is inhibitory to most of the nonacid formers.

III. Brilliant Green Lactose Bile Agar (12)

This medium is preferred because of the ease of determining the coliform type of colony. Subsurface colonies of the coliform group are deep red against a blue-green background. This medium is sensitive to light and preparation just prior to use is preferred. When this is not convenient the medium should be stored in the dark. For more complete identification representative colonies should be streaked on Levine's eosin methylene blue agar (31).

IV. Dextrose Agar (Acidified) (17)

This medium is preferred over malt agar (36), for detecting yeasts in fermenting vegetable brines [18] since it is more inhibitive to the lactic acid types of bacteria. Occasionally yeasts that will not grow on this medium are found in high salt concentrations, 15 to 20 per cent.[19] By reducing the tartaric acid to 3 ml per 100 ml growth can often be obtained. However, this modification should not be used where the salt concentration is known to be below 15 per cent, since acid-forming bacteria will grow sufficiently to make counting of yeasts very difficult.

Mold colonies are readily distinguished from yeasts on this medium, whereas differentiation of subsurface yeasts and film yeasts present more difficulty. Surface colonies of the common film-forming yeasts associated with pickle products and vegetable brines (i.e. Species of *Debaryomyces*, *Endomycopsis*, *Zygosaccharomyces*, *Candida* and *Pichia* [20, 21]) are generally dull and very rough as contrasted to the usually round, raised, white, glistening colonies of the fermentative, subsurface yeasts (i.e. species of *Torulopsis*, *Brettanomyces*, *Hansenula*, *Zygosaccharomyces*, and *Torulaspora* [22–24]). However, even where distinguishing colony characteristics of the 2 yeast groups exist they are not considered sufficiently clear cut for separation. Because of this, the procedure outlined under I, A (Film Yeasts) should be used. Film yeasts rapidly form a heavy, wrinkled surface film at one or both salt concentrations. Certain species, such as *Zygosaccharomyces halomembranis*, form heavier films at 10 per cent salt than at 5 per cent.[18, 21]

V. Liver Broth Plus Salt (33)

This medium has proved satisfactory for detecting obligate halophiles sometimes found in brined and dry-salted vegetables. The salt content of the medium should approximate that of the sample. No interference

has been encountered by growth of coliforms or yeasts in this medium. This is probably due to the inability of either group to initiate satisfactory early growth in laboratory media even at moderately high salt concentrations in competition with the very fast growing obligate halophiles.

VI. Liver Broth Medium (32)

This medium has proved useful in detecting saccharolytic and putrefactive mesophilic anaerobes. While a positive test is presumptive evidence of mesophilic, spore-forming, gas-producing anaerobes, of the butyric acid-forming types, more specific bacteriological tests are required on positive tubes before identification can be made. Also, spore formation in the sample may be neglible due to high acid production in the presence of readily fermentable carbohydrates, and even though previous activity by this group may have been quite high, negative results will be obtained in old brines. Positive results in this medium would indicate that these types of bacteria were responsible for the malodorous fermentation.

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CHAPTER IV

CANNED FOODS

The enormous quantity of canned foods that is manufactured and consumed makes it necessary that methods be available for the purpose of plant control, spoilage detection, guide to storage life, and general condition of products as consumed. This chapter is concerned with the canned food itself and does not attempt to go into methods for raw material, establishment of processing conditions, etc. Methods in this chapter differ from those in most other chapters in that actual enumeration of microorganisms is not carried out extensively as presence or absence of spoilage contamination is the main issue. Incubation at 37° C is carried out in most cases in the interest of obtaining results quickly instead of the 32° C incubation used for most of the other types of food.

I. GENERAL CONSIDERATIONS

Microbial spoilage of canned foods is due either to underprocessing or leakage.[1, 2] Underprocessing is the failure to destroy during the heat process all bacteria capable of subsequent growth in the product. Leakage is the contamination of the product after an adequate heat process by reason of a faulty seal.

A. Underprocessing

In spoilage due to underprocessing, there is a limited number of bacterial groups of importance and the limitation exists through the nature of the canning process. Products of low acidity, of which corn and peas are examples, are processed at high temperatures in steam under pressure. This procedure has the effect of eliminating all bacteria except those having spores of exceptionally high resistance to heat. Products of high acidity, of which tomatoes and fruits are examples, are inhibitory to most of the highly heat-resistant bacteria, and, consequently, much less severe heat processes need be used. In either case, the acidity (usually expressed as pH) of the food is an important factor in determining the type and character of spoilage.

B. Spoilage Related to pH

The spoilage relationships of the bacterial groups of importance based on a pH classification [3, 4] of the foods affected are as follows:

Acidity Classification	Typical Foods	Spoilage Organisms
Low acid pH 5.3 and higher	Peas Corn Lima beans Evaporated milk White potatoes	Thermophilic group: Flat-sour types Thermophilic anaerobes-gas producers Sulfide spoilage organism-H_2S gas producers Mesophilic group: Putrefactive anaerobes-gas producers Aerobic spore formers
Medium acid pH 4.5–5.3	Spinach Green beans Asparagus Sweet potatoes Beets	Same as for low acid products but thermophilic anaerobes assume importance over flat-sour types. Abnormal growth of putrefactive anaerobes.
Acid pH 4.5 and lower*	Tomato juice Pears Bananas Apple sauce Fruit preserves	Spore formers: Aciduric flat-sour types—*Bacillus thermoacidurans* Butyric anaerobes-gas producers Nonspore formers—Lactobacilli Yeasts Molds

C. Spoilage Manifestations

The spoilage manifestations exhibited by the principal spoilage types are as follows:

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* A recent study [5] has indicated that a pH of 4.6 may be used as the dividing line between medium acid and acid products.

	Type of Organism		Manifestations
Low acid and Medium acid products pH above 4.5	Flat sour	Can flat	Possible loss of vacuum on storage.
		Product	Appearance not usually altered. pH markedly lowered-sour. May have slightly abnormal odor. Sometimes cloudy liquor.
	Thermophilic anaerobe	Can swells	May burst.
		Product	Fermented, sour. Cheesy or butyric odor.
	Sulfide spoilage	Can flat	H_2S gas absorbed by product.
		Product	Usually blackened. "Rotten egg" odor.
	Putrefactive anaerobe	Can usually swells	May burst.
		Product	May be partially digested. pH slightly above normal. Usually typical putrid odor.
	Aerobic spore formers (odd types)	Can flat	Usually no swelling, except in cured meats when nitrate and sugar present.
		Product	Evaporated milk coagulated.
Acid products pH below 4.5	*Bacillus coagulans* or *Bacillus thermoacidurans* (flat-sour tomato juice)	Can flat	Little change in vacuum.
		Product	Slight lowering of pH. Off-odor and flavor.
	Butyric anaerobes (tomatoes, tomato juice and pears)	Can swells	May burst.
		Product	Fermented. Butyric odor.
	Nonspore formers (mostly lactic types)	Can swells	Usually bursts, but swelling may be arrested.
		Product	Acid odor.
	Yeasts	Can swells	May burst.
		Product	Fermented, yeasty odor.
	Molds	Can flat	Surface growth.
		Product	Musty odor.

D. Leakage

In spoilage resulting from leakage the organisms involved are heterogeneous free-living forms not subject to an orderly classification in relation to canned food groups. Leakage is generally indicated when mixtures of nonheat-resistant bacterial types and nonspore formers are encountered.

II. SAMPLING

A correct spoilage diagnosis may be a relatively simple matter or it may be quite complicated. Where the cause of spoilage is clear cut the examination of 6–12 cans may be adequate, but in some instances it may be necessary to examine as many as 50 cans before a decision can be reached as to cause of spoilage. An attempt should be made to obtain a representative sampling of the spoilage. Cans should be selected which do not have obvious defects and, if possible, buckled swells (mechanically damaged cans) should not be examined. Examination should be made also of normal cans and product for comparison to determine if spoilage may be progressive.

III. SPOILAGE TEST PROCEDURES

In any determination of spoilage causes certain historical, physical, and bacteriological data are essential.

A. Historical Data

From the historical standpoint one should ascertain as far as possible the following:

1. Extent of spoilage
2. Number of cans involved
3. Canning procedure used
4. Time and temperature of the process
5. Method of cooling
6. Any deviation from the normal canning procedure which might correlate with the spoilage outbreak.

B. Physical and Bacteriological Data

The physical and bacteriological data are obtained from the samples submitted. The various steps in obtaining the data are outlined as follows:

1. Preliminary Examination

a. Make set of records concerning the cans submitted including type of product, origin of sample and number submitted, make and size of container, code marks, label, and other pertinent information.

b. Physical examination of the can

NOTE AND RECORD—Condition of the can, mechanical defects, perforations, rust spots, dents, all other can abnormalities, (mark questionable points for later testing).

2. Preparation and Opening of Can

a. Arrange according to code lots. Number all cans to the right of the side seam at the can company end with can marking ink.

b. Clean can with soap and water, use naphtha, alcohol, or petroleum ether at site of opening if the can is greasy.

c. For sterilization at the site of opening, preferably grasp the container in the hand and invert over the flame of a Bunsen burner, distributing the heat with a circular motion over the previously cleaned top. Do not play the burner down upon the top of the can because this will result in a concentration of heat at the top, causing scorching of the material, and it might lead to spurting of the contents when the opening is made. The sterilization also causes a release of vacuum in the can, which will prevent any contamination that might result from an inrush of air when the opening is made. When containers are badly swollen, it is unwise to flame in the manner described. In such instances apply the treatment described by Fellers [6] and sterilize with bichloride of mercury (1:1,000). As an alternative, cleanse the site of the opening with 60 per cent alcohol. Open cans on the can company end, so as to preserve the packer's code and avoid disturbance of the seaming compound on the packer's end of the can.

d. Opening of container [7]

(1) For solid or semisolid products sterilize opener and cut a circular disc around a central puncture.

(2) For liquid products cut a hole about ½ in. in diameter with a sterilized tapered punch.

NOTE: These can openers are usually sterilized in the flame of a Bunsen burner.

3. Culturing of Can Contents [8, 9]

Remove solid products using sterile cheese triers, spoons or cork borers; remove semisolid or liquid products using sterile untapered glass tubes or pipettes.

Since the usual spoilage types of organisms are not especially fastidious in their growth requirements, and the spoiled product has already served as an enrichment medium, elaborate subculture media are unnecessary. Also, the counting of actual numbers is not essential as the main interest is in the presence or absence of contamination.

An example of a culturing procedure based on the nature of the product under examination is as follows:

a. Low and medium acid products (pH 4.5 and higher)

(1) *Culturing*

(a) Aerobic—Inoculate 4 tubes of dextrose tryptone bromcresol purple broth (22) with about 2 gm or ml of the food product per tube.

(b) Anaerobic—Inoculate 4 tubes of freshly boiled and cooled tryptone broth (67) with about 2 gm or ml of the food product per tube—stratify after inoculation with tryptone agar (66). If sulfide spoilage is suspected inoculate 4 tubes of sulfite agar (59) with 2 ml each.

(2) *Incubation*

Incubate half the number of aerobic tubes and half the anaerobic tubes at 37° C and the other half at 55° C for a period of 48–72 hr.

NOTE: Preheat all tubes intended for 55° C incubation to 55° C before incubating.

b. Acid products (pH 4.5 and lower) [10]

(1) *Culturing*

NOTE: Products with a pH of 4.5 or below are usually not pressure processed and depend for their preservation on the destruction of only the aciduric types capable of growing in the acid type product. In view of this, the subculture media must either be selective for the spoilage type involved or approach the normal pH of the acid product to eliminate the pos-

sibility of isolating viable organisms dormant in the product but capable of growing in a neutral subculture medium.

(a) Aerobic—Inoculate 2 tubes of orange serum broth (48) with 2 gm or ml of food product per tube. Incubate at 37° C for 72 hr.

(b) Anaerobic—Inoculate 2 tubes of either orange serum agar (47) or liver broth with crushed tomatoes (34) with about 2 gm or ml of food product per tube. Stratify the liver broth after inoculation with plain agar (49) or with an acid agar medium such as dextrose agar, acidified (17). Incubate under anaerobic conditions for 72 hr at 37° C.

(2) *Incubation*

Incubate the aerobic and anaerobic tubes at 37° C for 72 hr. There need be no incubation at thermophilic temperature unless indicated by history of the spoilage.

On occasion spoilage may be found in tomato juice in which the can remains flat but the product has an off-odor and flavor with or without a lowering of the pH. This is generally due to aerobic mesophilic or thermophilic spore formers. Spoilage of this type is one exception to the rule that products below pH 4.5 are immune to spoilage by spore formers. For this type of spoilage: [11]

Plate 1 ml and 0.1 ml of tomato juice, in duplicate, and pour with proteose peptone acid agar (52).

Incubate 1 set of plates at 37° C and the other at 55° C for 48–72 hr.

In rare cases spoilage of such products as tomatoes, pears, figs, pineapples, and nectarines show swelling of the cans and a butyric odor of the product. This is generally due to spore-forming anaerobes allied in their characteristics to *Clostridium pasteurianum*.[12-14] If these are suspected make a methylene blue stain (B7) from the tubes and examine under the microscope for spores and Clostridium-like organisms.

c. Concentrated Products

In concentrated acid products such as tomato paste or fruit pulp the spoilage may be highly localized. The top and the bottom of the product should be carefully examined for evidence of growth

of microorganisms before the body of the product is disturbed. Since the organisms causing the spoilage will be present in greater numbers than any dormant spore-forming types, a serial dilution technic should be used to dilute out the dormant type.

Mix the contents of the can thoroughly, prepare serial dilutions and culture and incubate as indicated under acid products above. The last dilution showing growth should contain a pure culture of the spoilage organism.

4. Supplementary Tests

a. Direct microscopic examination

Sometimes all the organisms causing deterioration die during storage and bacteriological diagnosis will depend almost entirely on the microscopic examination. An idea of the types of organisms involved will often be obtained only by this examination.

Prepare smears on glass slides by transferring liquid material with a platinum loop. If material is solid add a small portion to a drop of water on the slide, mascerate and then make a thin smear elsewhere on the slide. Fix with heat and stain with gentian violet stain (B2) or methylene blue stain (B7). Do not use Gram's stain at this point.

b. In order to demonstrate spore formation, subculture from the aerobic broth tubes on nutrient agar slants (45) and incubate at 37° C and 55° C for 72 hr to determine spore formation:

(1) Make smears from the agar slant and dry slide over a flame.
(2) Flood with aqueous malachite green stain (B6) for 30–60 sec and then heat to steaming 3 or 4 times.
(3) Wash off excess stain in running water.
(4) Immerse in aqueous safranin stain (B9) for about 30 sec.
(5) Wash, blot, dry, and examine.
The spores will stain green and the vegetative cells red.

c. Make pH determination on product in all cans using a glass electrode.

d. Examine product to determine condition, appearance and odor. Never taste spoiled foods. Note especially any uncooked condition. These observations may be important later in determining type and cause of spoilage.

e. Determine net and drained weight on leafy vegetables such as spinach, mustard greens, etc. These are important as overfilling diminishes the rate of heat penetration during processing and may lead to spoilage. Drained weight determination on badly spoiled leafy products may be omitted.

f. Make quantitative analysis for iron in dark spinach and beets. (See under unusual types of spoilage.)

5. Can Examination

For proper evaluation of can defects cans must be examined by a person thoroughly conversant with the essential indications of seam abnormalities. Unless the laboratory examiner possesses these necessary attributes, he should limit his observation of the cans to those defects readily discernible as indicated in the Preliminary Examination under Physical Examination of the Can, III, B, l, b. General information regarding cans and their performances can be obtained from the literature.[15–21] A representative number of nonspoiled cans should be examined for comparison with spoiled cans.

6. Gas Analysis

Where the can is swollen but the microbial count is low it is desirable to analyze the gas for detection of hydrogen swells. This nonbacterial type of spoilage gives a combustible gas and the inside of the can shows corrosion, whereas the gas from bacterial spoilage is predominately carbon dioxide. The combustible nature of the gas can be demonstrated by making a small puncture in the can while being held adjacent to a flame or a differential analysis can be made in an orsat type of gas analyzer.

IV. INCUBATION TESTS

Unspoiled cans may be subjected to incubation tests to determine keeping quality or degree of sterility. It is often desirable to incubate cans of food as an acceleration test to determine the possibilities of spoilage during storage.

A. Sampling

The sample for incubation should consist of at least 24 cans with 1 can selected from each of 24 different cases. More than 24 cans is sometimes required if the incidence of spoilage is very low.

B. Scheme of Incubation

The temperature and period of incubation is determined by the pH of the product and the experience of the canning industry in making such tests.

1. Low-Acid Foods (pH 4.5 and higher)

Incubate the cans for 14–30 days at 37° C and for 7–10 days at at 55° C.

EXCEPTION: No incubation at 55° C is necessary for meat and fishery products. However, meat products should be incubated at 55° C if cereals are present. Government meat inspection regulations [22] require that representative samples of meat be incubated at least 10 days at 98° F (37° C).

2. Acid and High-Acid Foods (pH 4.5 and lower)

Incubate the cans for 14 days at 37° C. No incubation is carried out at 55° C. Examination may be direct if 14 days have elapsed since packing and the temperature has been about 30° C.

EXCEPTION: Incubate whole tomatoes at 55° C for certain rare thermophilic spoilage types if indicated, or at 32° C if butyric anaerobes are suspected.[12]

C. Incubation Procedure

1. Check all cans for obvious defects, place half of the cans at 37° C, and the other half at 55° C if thermophilic incubation is indicated.

2. Incubate as indicated above. Examine the cans at frequent intervals during the incubation period—remove "swells" and examine as under Spoilage Test Procedures, III.

3. Open all flat cans at the end of the incubation period. Observe condition of contents, test for "flat sours" with a glass electrode pH meter. Certain indicators (such as bromcresol purple) may be used to test for "flat sours" (in corn and peas) if desired.

4. Should spoilage be detected above, examine as under III, Spoilage Test Procedures.

V. STERILITY TESTS

Unspoiled cans may be examined bacteriologically to determine the presence of viable but dormant organisms. The procedure is identical

with that used in the examination of spoiled cans except that the quantity of material subcultured should be increased as much as possible and numerous controls should be used. Fifteen to 25 gm should be used if possible. Care should be exercised in interpreting the results since adventitious contamination may occur.[23] If necessary to check for air contamination place 2 open Petri plates of solidified agar immediately adjacent to the can at the time of sampling. If it seems desirable to obtain inocula from component parts of unspoiled meat products, e.g., fat-liquid-meat, special procedures based on preincubation at a relatively high temperature may be used.[24] This can be accomplished by placing cans at 50°–55° C for 1–2 days and then chilling immediately. The fat wil be trapped at the top as a solid component.

VI. INTERPRETATION OF RESULTS

Spoilage Diagnosis

A. Usual Types of Spoilage

1. Underprocessing

a. *Low-Acid Products*

The presence of only spore-forming organisms growing at 37° C and/or 55° C is indicative of underprocessing. In some cases, it is necessary to use a variety of media to determine spore formation. Spoilage by thermophilic anaerobes[25] is indicated by gas in the anaerobic tubes at 55° C and a cheesy odor in the tube. Rods are found upon microscopic examination. Spoilage by putrefactive anaerobes[26] is indicated by gas in the anaerobic tubes at 37° C, rods and spores are found upon microscopic examination, and the tubes have a putrid odor. Spoilage by mesophilic and/or thermophilic flat-sour types[27] is indicated by acid production in the aerobic broth tubes at 37° C and/or 55° C.

b. *Acid Products*

Spoilage in acid products is generally caused by nonspore-forming lactobacilli[28, 29] and yeasts. However, on occasion spoilage of tomato juice[30] has occurred in which the can remains flat but the product had an off-odor, and taste with or without a lowered pH, due to aerobic, mesophilic, or thermophilic spore-formers. Spoilage of this type is an exception to the general rule

that products below pH 4.5 are immune to spoilage by spore-formers. The only other exception is spoilage occasionally encountered in products such as tomatoes, pears, figs, pineapples, and nectarines due to spore-forming anaerobes, allied in their characteristics to *Clostridium pasteurianum* [12-14] in which the can swells and the product has a butyric acid order. Spoilage in acid products has a butyric acid odor. Spoilage in acid products due to thermophilic anaerobes is very rare.

2. Gross Underprocessing

Occasionally a crate of cans may bypass the retort room without receiving any heat process. Nonspore-forming as well as spore-forming organisms are usually observed in the cultures of such samples. While most of the spoilage characteristics are similar to those of spoilage due to leakage it can generally be distinguished by the uncooked texture of the product. Such spoilage is also limited to a single batch.

3. Leakage

The presence of a mixed flora of rods and cocci on microscopic examination and the isolation of these organisms on subculture is indicative of *leakage*. The diagnosis is generally confirmed by the can examination indicating leakage. However, the can examination may not always confirm the bacteriological findings, and the diagnosis may have to be made on the basis of other findings or information. The cause may be found in excessive contamination of cooling water, or slight damage to cans through rough handling, e.g., can unscramblers, rough conveyor systems, etc.

4. Precanning Spoilage

In some instances, spoilage may result from the growth of microorganisms in the food before canning. In this type of spoilage a mixture of bacterial types is usually observed microscopically from normal appearing containers, but none of the organisms are viable. The pH, odor, and appearance may be abnormal.

5. Hydrogen Swells

If no microorganisms can be demonstrated in smears or cultures of swelled cans and the product appears normal there is a possibility

that the swelling of the cans is due to the production of hydrogen by chemical action of the contents on the containers.[31] Gas analysis should be made and the interiors of the cans examined for evidence of corrosion. The proportion of hydrogen in the gas from a hydrogen swell varies with the length and temperature of storage. While hydrogen swells are usually associated with acid foods, cans of low-acid foods may also become hydrogen springers if packed with initially low vacuum and stored for an excessive period of time.

6. Key to Diagnosing Cause of Usual Bacterial Spoilage

The following chart serves as a general guide in diagnosing the causes of bacterial spoilage of low-acid and medium-acid products.[32]

	Underprocessing	Leakage
Can	Flat or swelled seams generally normal	Usually swelled; may show defects
Product		
Appearance	Sloppy or fermented	Frothy fermentation; viscous
Odor	Normal, sour, or putrid, but generally consistent	Sour, fecal, generally varying can to can
pH	Usually fairly constant	Wide variation
Microscopic and cultural	Cultures of spore-forming rods. Growth at 37° C and/or 55° C may be characteristic on special media.	Mixed cultures, generally rods and cocci. Growth only at usual temperatures.
History	Spoilage usually confined to certain portions of pack	Spoilage scattered

In acid products diagnosis may be less clearly defined than in low-acid or medium-acid products—similar organisms may be involved in both underprocessing and leakage. The picture is sometimes confused because in some cases, a few cans may leak though the majority show spoilage due to underprocessing.

B. Unusual Types of Spoilage

Spoilage which does not fit into the general scheme may occur in rare instances and are considered separately as unusual types. It is advisable to consult the literature for the details of the original investigation.

1. Black Beets [33]

Spoilage of this type in canned beets is characterized by a deep purple to black discoloration and an increase in pH. It is caused by an aerobic mesophilic spore former requiring an abnormally high amount of iron to develop the color. The can remains flat.

2. Mold in Blueberries [34]

This defect shows small plaques of mold on the surface of blueberries canned in enamel-lined cans. No growth is found in plain cans due to rapid take up of oxygen by the metal. Oxygen consumption is delayed in the enameled cans sufficiently to permit minimal growth of a mold of unusual heat resistance.

3. Swelling in Canned Chopped Ham [35]

This is due to the growth of aerobic spore-forming organisms in the presence of a nitrate-nitrite, sugar combination. This type of spoilage can be confirmed by the use of Jensen's pork-sucrose-nitrate medium(28).

4. Coagulation of Evaporated Milk [36, 37]

This defect is due to aerobic spore-forming organisms identical with organisms producing flat-sour tomato juice. The can remains flat.

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CHAPTER V

CARBOHYDRATE PRODUCTS

Carbohydrate materials are among the most commonly used ingredients in the manufacture of food products. Because of their nature and origin, prepared sweetening agents may be contaminated with a variety of bacteria, yeasts, and molds and, therefore, serve as a potential source of contamination of products in which they are used.[1, 2] The need for uniform methods for the bacteriological examination of carbohydrate products has long been recognized as an aid to producers and users of carbohydrate materials in the manufacture of high quality products. The methods presented will be useful in determining the presence of those types of microorganisms indicative of insanitary conditions of manufacture and handling or those contaminants which may cause trouble in foods in which the carbohydrates are used as ingredients.

CLASSIFICATION

For the purpose of presenting methods for their examination carbohydrate products are divided into 3 main groups as follows:

I. Sugars

- A. Dry (sucrose, dextrose, levulose, maltose, lactose, etc.)
- B. Liquid (invert sugar, liquid sugar, etc.)

II. Sirups and Molasses

- A. Natural products (honey, maple sirup)
- B. Manufactured or processed products (corn and other grain sirups, blends, molasses, etc.)

III. Starches and Other Complex Carbohydrates (corn, wheat, tapioca starch, and dextrin)

COLLECTING AND HANDLING OF SAMPLES[3, 4]

Carefully cleansed, rinsed, dried, and sterilized sample bottles or cans of approximately 1-qt capacity should be used. For dry products take

at least ½-lb portions from each of 5 bags or other containers of the lot and send to the laboratory for examination.

For liquid sugars, sirups, and molasses use sterile sample bottles of 1-qt capacity and obtain 1 pt from each of 4 pails, barrels, or other bulk containers of the lot. If the product is in package-sized containers withdraw 5 packages from the lot and forward to the laboratory intact.

Care must be taken to select samples representative of the lot. Take precautions to handle samples in such a manner as to prevent contamination or change prior to the examination. It is not necessary to ship under refrigeration, but it is important to prevent the absorption of atmospheric moisture. Examine the samples as soon as possible after receipt at the laboratory.

MICROBIOLOGICAL METHODS

I.A. Dry Sugars

These methods are recommended for the examination of dry sugar for mesophilic and thermophilic types of organisms. A wide variety of mesophilic types may be encountered in carbohydrate material; however, relatively few types are considered to be significant from the standpoint of food spoilage and sanitary practices. The important types are groups of acid and nonacid-forming bacteria and yeasts and molds which are enumerated by the use of selective media.

Three types of food spoilage bacteria are considered particularly significant when carbohydrate products are used as ingredients in other types of food, namely: *Bacillus stearothermophilus*, causing flat-sour spoilage; *Clostridium thermosaccharolyticum*, a thermophilic anaerobe not producing hydrogen sulfide; and *Clostridium nigrificans*, producing hydrogen sulfide spoilage.

Weigh 20 gm of representative sample of sugar into a sterile 150-ml Erlenmeyer flask, marked to indicate a 100-ml volume, and add sterile distilled water to 100-ml mark: Dissolve and shake thoroughly, and distribute 5-ml aliquots into 2 sets of 5 Petri plates.

1. Total Count

For the enumeration of mesophilic bacteria pour 1 set of 5 plates with dextrose tryptone bromcresol purple agar (21). Incubate for 72 hr at

32° C. Count all the colonies on the 5 plates and multiply by 5, since the combined plates represent 2 gm of sugar, and report as the "total count per 10 gm of product." Also, make differential count of acid and nonacid producers by means of observing the yellow halo around the acid colonies.

2. *Yeasts and Molds*

For the enumeration of yeasts and molds pour the other set of 5 plates with potato dextrose agar, acidified (51), or dextrose agar, acidified (17). Incubate for 5 days at 32° C. Count at 3 days if molds are likely to overgrow the plates. Make a differential count of the 5 plates and report as the number of yeasts and molds per 10 gm of product, by multiplying the combined count by 5.

3. *Thermophilic Spores* [5]

To determine the number of spores of thermophilic bacteria bring the above solution to boiling and boil for 5 min. Replace evaporation with sterile water.

a. *Flat-Sour Type*

Place 2 ml of the boiled sugar solution in each of 5 Petri plates and pour with dextrose tryptone bromcresol purple agar (21). As soon as solidified preheat to 55° C and incubate 48 hr at 55° C. Count the colonies on the 5 plates having yellow halos around the colony, multiply by 5, and report as the flat-sour spore count per 10 gm of sugar.

b. *Thermophilic Anaerobic Spores Producing Hydrogen Sulfide*

Distribute 20 ml of boiled sugar solution equally among 6 freshly exhausted tubes of sulfite agar (59). Preheat the tubes to 55° C and incubate 72 hr at 55° C. Count the number of black colonies in the 6 tubes, multiply by 2.5, and report as number of thermophilic anaerobic spores producing hydrogen sulfide per 10 gm of sugar.

c. *Thermophilic Anaerobic Spores Producing Gas But No Hydrogen Sulfide*

Distribute 20 ml of the boiled sugar solution equally among 6 tubes of liver broth medium (32). Stratify the tubes with a layer

of petrolatum or with nutrient agar (45). Preheat to 55° C and incubate at 55° C for 72 hr.

This method does not permit true enumeration of organisms so report results as number of positive tubes showing gas.

I.B. Liquid Sugars

Weigh 20 gm of liquid sugar into a sterile 150-ml Erlenmeyer flask and make up to the 100-ml mark with sterile distilled water.

Proceed with the examination in accordance with each step found under I, A, Dry Sugars. Report results as numbers of organisms per 10 gm of liquid sugar.

II. Sirups and Molasses

A. *Natural Products*

Stir the sample of honey or maple sirup thoroughly. Weigh 11 gm of product into a 99-ml dilution blank (23A) and shake 50 times through a 1-ft distance. Make serial dilutions by transferring 1-ml aliquots to 9-ml dilution blanks or 11-ml to 99-ml dilution blanks (23A).

1. Total Count

Transfer appropriate aliquots of dilutions to Petri plates, in duplicate, and pour with dextrose tryptone bromcresol purple agar (21). Incubate 72 hr at 32° C. Count colonies on plates and report average as total count per gm of product. Differential count may be made.

2. Yeasts and Molds

Most spoilage in these products is caused by yeasts and molds.[6, 7]

Transfer appropriate aliquots of dilutions to Petri plates, in duplicate, and pour with potato dextrose agar, acidified (51), or dextrose agar, acidified (17). Incubate for 5 days at 32° C. Count at 3 days if molds are likely to overgrow the plates. Make a differential count and report the average as the number of yeasts and molds per gm of product.

B. *Manufactured or Processed Products*

1. If the examination is for the purpose of detecting presence of organisms which might cause spoilage in the product, proceed as under II, A above.

2. If the material is to be used as an ingredient in other food products and the examination is for potential spoilage organisms, proceed as under I, B and I, A.

III. Starches and Other Complex Carbohydrates

Where such products are to be used as ingredients in other foods it is valuable to know the population of certain potential spoilage organisms. Weigh 11 gm of product into a 99-ml dilution blank with sand (23B). Shake vigorously for 2 min. Allow to stand briefly for any heavy particles to settle and prepare serial dilutions.

1. Total Count

Transfer 1-ml portion of appropriate aliquots of dilutions to Petri plates, in duplicate, and pour with dextrose tryptone bromcresol purple agar (21).

Incubate at 32° C for 72 hr. Make total and differential count of acid and nonacid formers and report as numbers of organisms per gm of product.

2. *Anaerobic Total Count*

Transfer 1-ml portion to duplicate oval test tubes, as presented under cereals and cereal products, Chapter VI, Section II, and use total plate count agar (64) as a culture medium.

After thorough mixing chill in cold water to solidify the agar and fill the round necks of the tubes with reduced methylene blue agar (44).

Incubate at 32° C for 72 hr. Count colonies with a hand lens and report as number of anaerobic colonies per gm of product.

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3. *Thermophilic Spore Count*

a. *Flat-Sour Type*

Into 100 ml of melted dextrose tryptone bromcresol purple agar (21), transfer 20 ml of the 1:10 dilution, or primary dilution above. Mix thoroughly and shake in boiling water for about 3 min and then place in flowing steam for 30 min with occasional shaking. Distribute equally between 5 Petri plates.

Incubate at 55° C for 72 hr. Make a count of all colonies showing a yellow halo. Multiply the combined count of the 5 plates by 5, and report as the "flat-sour spore count" per 10 gm of product.

b. *Thermophilic Anaerobic Spores Producing Hydrogen Sulfide*

Pipette 20 ml of the above 1:10 dilution equally among 6 tubes of melted sulfite agar (59). Shake the tubes in boiling water for about 3 min to get good distribution and then heat in flowing steam for 30 min. Solidify the agar in the tubes. Preheat to 55° C and incubate at 55° C for 72 hr. Count the number of black colonies in the 6 tubes, multiply by 5, and report as number of spores producing hydrogen sulfide per 10 gm of product.

c. *Thermophilic Anaerobic Spores Producing Gas But No Hydrogen Sulfide*

Distribute 20 ml of the above 1:10 dilution equally among 6 tubes of preheated liver broth medium (32). Mix gently while holding the tubes in boiling water for about 3 min and then hold in flowing steam for 30 min. Cool and stratify with petrolatum or nutrient agar (45).

Preheat to 55° C and incubate at 55° C for 72 hr. Report results as the number of tubes showing gas.

INTERPRETATION OF RESULTS

The interpretation of results of microbiological examination of carbohydrate materials is difficult due to the wide variety of uses to which these products lend themselves. High counts of flat-sour producing types of sporulating bacteria in carbohydrate materials are of comparatively little interest to the producer of bakery products, while they are of great interest to the user of these products in canned products.

Therefore, in order to interpret results in terms of significance to particular products it is necessary for the laboratory worker to consult references which relate to particular products in which carbohydrate materials are used as ingredients.

For general information on carbohydrate materials see Thaysen, et al.,[8] and Hucker, et al.[9] For information on significance for canned products see [1, 10–11]; for meat products see Jensen,[12] and for other products.[6, 7, 13]

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CHAPTER VI

CEREALS AND CEREAL PRODUCTS

The term, cereal products, covers a wide field for it may be considered to include all cereal products from the unmilled grain to any manufactured products in which cereal products are significant ingredients. Here the term is restricted to its more common usage, that of food products consisting essentially or wholly of materials obtained from milled grain; the term, cereals, is used as a synonym for unmilled grain. Even this list is long and calls for a variety of bacteriological procedures.

In addition to the usual bacterial counts on cereals and cereal products it is also frequently necessary to determine numbers of specific types of organisms present as Tanner [1] has emphasized. For example, it is important for a canner to know whether spoilage organisms are present in cereal products he uses and, if so, what types are present.[2] In the baking and milling fields [3, 4] significance of "rope-producing" bacteria has been repeatedly demonstrated. In fact, many food processors buy cereal products on the basis of microbiological specifications. It is likewise of interest to most food processors to note that development of rancidity [5] and of mustiness [6] in wheat flour have been shown to be associated with its bacterial and fungal flora. Tanner [1] has also pointed out that public health officials may be concerned at times as to whether pathogenic or food poisoning bacteria may be present in cereal products.

SAMPLING

If possible, obtain original containers of cereal products and send them intact to the laboratory for examination. If material is in large or bulk containers, obtain representative sample by mixing or by sampling various parts of the mass. Take aseptic precautions in opening such containers so as to avoid contamination by dust which may have settled at the point of opening. Use sterile, dry spoons, scoops, triers, etc., and place samples in sterile, dry glass jars or metal cans, of such size as to accommodate an adequate sample of the product. The inadequacy of

results, based on a single sample and the need for testing an adequate number of samples, has been repeatedly emphasized.[7]

If small packages are available, select several from various parts of the lot and make a composite sample in the laboratory.

PREPARATION OF DRY SAMPLE

Remove any dust from the surface of the package with a slightly moistened cloth and then aseptically open the package and remove the sample. Make a representative composite sample from several of the packages.

Since most cereal products are insoluble in water, the preparation of suitable dilutions for inoculating Petri plates offers a problem. In order to obtain uniform and accurate results it is necessary to use comparatively large samples and to have them in a rather finely divided state, e.g., flour or meal. In dry or solid cereal products distribution of the bacterial population is frequently not uniform, hence the necessity of using comparatively large amounts to obtain representative samples.

The smaller the particle size of material the greater the chance of bacteria being exposed on the surfaces and being washed off into the dilution water. This process may be aided by the abrasive action of sand, glass beads, etc., incorporated in dilution blanks. If sample is in the form of a flour it is tested without further treatment, but if coarse, the particle size of the representative sample should be reduced to approximately that of a coarse meal by grinding, with aseptic precautions, in a sterile mortar and pestle, or other sterile grinding equipment.

If the cereal sample consists of whole grain rice, it may be tested without grinding, as tests have shown no difference in count when whole rice was used or when the sample was ground.[8] However, with other grains such as wheat, which have creases or other irregular surfaces, it is necessary to grind them before testing.

MICROBIOLOGICAL EXAMINATION

After thoroughly mixing the prepared sample, weigh aseptically 11 gm in a sterile aluminum boat or on a piece of sterile paper. Transfer to a dilution bottle, fitted with a solid stopper, containing 99 ml of sterile water and approximately 10 gm of purified sterile sea sand (23B).

Shake vigorously for at least 2 min. Allow to stand for 2 or 3 min or until most of the larger solid particles have settled to the bottom. This constitutes the primary dilution.

I. Aerobic Total Plate Count

From the supernatant fluid of the primary dilution, transfer 1-ml portions to Petri plates in duplicate. After again shaking and allowing to settle prepare higher serial dilutions using dilution blanks with sand (23B). Inoculate Petri plates in duplicate with 1 ml from each dilution and pour with total plate count agar (64).

Incubate the plates at 32° C for 3 days and count the colonies. Report the results as the total plate count per gm of product.

II. Anaerobic Total Count

From the above serial dilutions transfer 1-ml aliquots to Petri plates and pour with total plate count agar (64). Incubate under anaerobic conditions for 3 days at 32° C. Oval test tubes, modified by a round neck, may be used instead of Petri plates.[9] Use tubes of recently melted and rapidly tempered agar (to minimize the reabsorption of oxygen). Immediately after inoculating tubes and twirling them to mix the inocula and the medium, chill in cold water to solidify agar. Promptly upon solidification fill the round necks of the tubes 2/3 full with reduced methylene blue agar (44).[9, 10] This "seal" or "indicator cap" of agar excludes oxygen from the inoculated medium during incubation and by its change of color indicates whether oxygen has penetrated into the inoculated medium. Thioglycolate agar (60) may also be used.

Incubate tubes 48–72 hr at 32° C and count colonies. A Petri plate counter is helpful in counting, particularly if modified by a rectangular slot to accommodate the tube.

Report results as numbers of anaerobic colonies per gm of material.

If inocula produce much turbidity, or if growth produces so much gas that it shatters the agar and the colonies in tubes can not be counted. the following alternate prodecure may be used:

A. *Alternate Procedure*

Inoculate tubes of freshly deoxygenated liver broth medium [11, 12] (32) in duplicate from serial dilutions prepared above. Stratify tubes of

liver broth medium with nutrient agar (45) and incubate at 32° C for 5 days. If gas is not formed beneath the agar a microscopic examination of stained smears of supernatant liquid in the tubes should be made as turbidity can not always be relied upon to denote growth.

The anaerobic count obtained by the alternate method is estimated to be in the range shown by the reciprocal of the highest dilution showing positive growth and the next higher dilution. If a closer estimate is desired than is given by the usual decimal dilution technic, lower multiples may be used in making serial dilutions.

III. Thermophilic Counts (55° C)

Thermophiles which are frequently of importance, particularly to canners, are of certain specific types, methods for which are given under subsequent headings.

A. *Aerobic Total Count**

This count is made in the same manner as the aerobic total plate count using total plate count agar (64), except that plates are incubated at 55° C for 48 hr. It is desirable to determine these 2 types of plate counts at the same time, as thereby some indication is obtained as to whether the aerobic thermophiles are obligate or facultative. It is to be noted, however, that some bacilli, particularly mesophiles with a high maximum temperature range, may show some colonial development while plates are warming up in the 55° C incubator. Precautions should be taken to hold this warm-up period to a minimum. Report results as total aerobic thermophilic colonies per gm of cereal product.

B. *Anaerobic Total Counts**

For the total anaerobic counts use the oval test tube method described under II.[9] Prepare tubes in the same manner as for 32° C anaerobic count, except that the modified oval tubes are incubated at 55° C for 48 hr.

C. *Alternate Procedure*

If, due to turbidity, colonies in the agar tubes cannot be counted satisfactorily, the alternate method described under II, A, may be used. Incubate the tubes of liver broth medium (32) at 55° C for 48 hr.

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* Note: The term, "total count," is not used here in its strict technical sense, but according to usage of the canning industry for an undifferentiated count of all thermophilic organisms that develop on a general medium of good productivity.

IV. Thermophilic Spore Counts

1. Preparation of Dilutions for Inocula: Weigh 11 gm of sample aseptically and put into a sterile 99-ml dilution blank (23A). Mix this 1:10 dilution thoroughly and use it for pipetting into the various differential media for detecting various specific types of thermophilic spore formers.[13]

A. *Aerobic Total Spore Count* *

The total aerobic thermophilic spore count is made by pipetting 20 ml of the 1:10 cereal suspension into a flask containing 100 ml of dextrose tryptone agar (20)[14] held at a temperature of 55°–60° C.[12, 15] Use large bore pipettes and keep the cereal suspension under constant agitation during the pipetting operation. After the cereal suspension has been added to the agar, shake the flask in boiling water for approximately 3 min and then heat in flowing steam (Arnold sterilizer) for 30 min with occasional shaking. Distribute heated cereal-agar mixture equally between 5 Petri plates. Incubate at 55° C for 48 hr.

The combined count from the 5 plates represents the number of aerobic thermophilic spores in 2 gm of product. Multiply this count by 5 to express the results in terms of number of spores per 10 gm of sample. When plates are so heavily seeded as to make counting impractical, prepare higher dilutions in the agar as described above.

B. *Flat-Sour Type Count*

Pipette 20 ml of the 1:10 cereal dilution into 100 ml of melted dextrose tryptone bromcresol purple agar (21),[14] stirring the medium as cereal dilution is added.[13] Use pipettes with large bore and keep cereal suspension agitated during pipetting procedure. Shake agar-cereal mixture in boiling water for 3 min and then heat in flowing steam (Arnold sterilizer) for 30 min with occasional shaking. Pour equal amounts into each of 5 Petri plates. When inoculated medium in the plates has hardened to a maximum degree stratify with a thin layer of plain agar (49) to combat spreader type of colonies. Incubate plates at 55° C for 48 hr. Make readings at end of 24 and 48 hr. Flat-sour colonies are characteristic in appearance, being round, 2–3 mm in

* NOTE: The term, "total count," is not used here in its strict technical sense, but according to usage of the canning industry for an undifferentiated count of all thermophilic organisms that develop on a general medium of good productivity.

diameter, with an opaque central "spot," and usually surrounded by a yellow halo.

Combine the count of the 5 plates and report result as number of flat-sour spores per 10 gm of cereal.

C. *Anaerobic Spores Producing H_2S (Sulfide Spoilage)*

Pipette and equally distribute 20 ml of the 1:10 cereal dilution [13] between 6 tubes of melted sulfite agar (59). Shake inoculated tubes thoroughly to distribute cereal and heat in flowing steam (Arnold sterilizer) for 30 min with occasional agitation. After heating agitate tubes thoroughly, allow agar to solidify, warm to 55° C and then incubate for 5 days at 55° C. The sulfide-producing organisms produce typical black colonies in sulfite agar. As many anaerobes are able to produce H_2S, difficulties in differentiation of types may be encountered, especially with samples containing an abundance of thermophilic anaerobes. The atypical types may be differentiated by marking the colonies after 72 hr incubation and continuing the incubation another 48 hr, as during continued incubation the atypical colonies tend to produce enough gas to break up the medium.[12, 13]

Count the colonies in the 6 tubes and report as the number of sulfide-producing anaerobic thermophiles per 10 gm of cereal product.

D. *Anaerobic Gas-Producing Spores*

Pipette 20 ml of the 1:10 cereal dilution and distribute equally among 6 tubes of liver broth medium (32),[11] which have been previously heated to 100° C to exhaust the oxygen.[13] After agitating to distribute cereal thoroughly and prevent excess foaming, heat tubes in flowing steam (Arnold sterilizer) for 30 min, then cool and stratify with plain agar (49). Preheat tubes to 55° C and incubate at 55° C for 5 days.

Report results as the number of tubes showing gas.

V. Yeasts and Molds

Yeasts occur in flour [7] similarly to their occurrence in wheat,[15] namely, that their numbers vary erratically with little evidence of consistency with relation to other types of microorganisms present. Such yeasts may affect fermentation during the leavening process.

From the dilution blanks prepared for total plate counts transfer 1-ml portions, in duplicate, to Petri plates and pour with potato dextrose agar (acidified) (51), malt agar (36), or malt-salt agar (39). Incubate at 32° C for 3 and 5 days and count yeast and mold colonies developing on plates. Report average count as yeast and mold colonies per gm of product.

The salt content of malt-salt agar is sufficient to inhibit growth of bacteria commonly present in flour.[16] Comparative tests indicate that while common molds grow rather slowly on this medium those common to flour grow quite rapidly.

VI. "Rope" Spore Count

Prepare 2 different dilution blanks for this test as follows; (a) place 400 ml of 0.5 per cent NaCl solution in a rubber stoppered 16-oz bottle containing 20 gm of clean sea sand and (b) place 100 ml of 0.5 per cent NaCl solution in a rubber stoppered 4-oz bottle. Sterilize the 2 types of dilution bottles for 30 min at 121° C (15 lb pressure).

Weigh 20 gm of cereal product and transfer to the 400-ml dilution blank. Shake vigorously for 2 min. This constitutes the primary suspension.

From the primary suspension inoculate 1-ml and 2-ml amounts, in duplicate, into test tubes of Amos and Kent-Jones medium (3).[3] After reshaking suspension transfer 20-ml to a 100-ml dilution blank, (b) above, and shake vigorously. From this dilution blank inoculate, in duplicate, tubes of the same medium with 4, 3, 2.5, 1.7, 1.5, 1.3, 1.2, and 1-ml amounts, respectively. The bottle should be shaken before each withdrawal to keep the cereal product in even distribution.

Mix the contents of test tubes thoroughly and place immediately in a bath of boiling water so that the level of water is above the liquid in the tubes. An Arnold sterilizer may be used but the cotton plugs must be protected from water. Heat for 20 min, shake tubes thoroughly, and place in 32° C incubator. Incubate for 48 hr the contents of the tubes being mixed 3 or 4 times during first 24 hr of incubation.

Record as positive those tubes showing a pellicle on the surface. The estimated number of rope spores per gm of material is taken as the

reciprocal of the smallest fraction of a gm of product giving a positive result.

The quantities of cereal corresponding to the various inocula used above are:

2.0 ml	primary	suspension	= 1/10	gm	cereal
1.0 ml	"	"	= 1/20	"	"
4.0 ml	second	suspension	= 1/30	"	"
3.0 ml	"	"	= 1/40	"	"
2.5 ml	"	"	= 1/48	"	"
2.0 ml	"	"	= 1/60	"	"
1.7 ml	"	"	= 1/70	"	"
1.5 ml	"	"	= 1/80	"	"
1.3 ml	"	"	= 1/92	"	"
1.2 ml	"	"	= 1/100	"	"
1.0 ml	"	"	= 1/120	"	"

If the cereal contains more than 120 rope spores per gm, higher dilutions must be prepared.

If a negative result is obtained in a tube containing a greater quantity of cereal than the amount showing a positive result, then the estimated number of spores is taken as the reciprocal of quantity next greater than smallest amount of cereal giving a positive result.

It may be desirable to determine if the pellicles are formed by rope-producing organisms of the *B. mesentericus* group or nonrope-producing members of the *B. subtilis* group. Inoculate agar slants from pellicles and observe resultant growth. Members of the *B. mesentericus* group produce gray-white growth that is moist and blister-like at first, becoming drier and finely wrinkled; whereas, the *B. subtilis* group produces a whitish, spreading growth, the edges appearing like an interwoven mass of fine hairs, when viewed under a low-power lens.[3]

A. *Alternate Method* [17]

Dispense 20-ml quantities of the Amos and Kent-Jones medium (3) in 25 mm x 125 mm test tubes and sterilize for 30 min at 121° C (15 lb pressure). Incubate these tubes for 48 hr and discard any showing growth.

Prepare and sterilize dilution bottles containing 150 ml of water and a spoonful of glass beads.

Weigh 1.5 gm of cereal product into a dilution blank to give a 1:100 dilution. Shake well and heat in boiling water for 20 min with frequent shaking.

From a water blank withdraw 15 ml of solution and discard it. Remove the 1:100 dilution from water bath and after shaking transfer 15 ml to second dilution blank to make a 1:1,000 dilution.

From the 1:100 dilution bottle, after shaking, inoculate 5 test tubes of test medium with 10 ml each of the solution.

From the 1:1,000 dilution bottle inoculate 5 test tubes with 10 ml each, and 5 test tubes with 1 ml each. To the latter test tubes add 9 ml of sterile water to maintain constant volume in all inoculated tubes.

Incubate test tubes for 24–48 hr at 32° C. Record number of tubes, in each series, showing pellicle growth. From this information determine most likely number of spores per gm of material by referring to "Table of Most Probable Numbers," Table 1, Chapter XVI.

VII. Bacilli Forming Gas in Presence of Nitrates

If the cereal product is for use in processed foods containing nitrates, such as bologna, meat loaf, certain canned meats, etc., it should be examined for presence of bacilli that normally are not classified as gas-formers, but which do form gas in the presence of nitrates.

From each of the dilution blanks prepared for the "total plate count," I, inoculate 5 tubes of Jensen's pork sucrose nitrate medium (28) [18] with 1 ml of suspension. Introduce inoculum at the botton of the medium in tube taking care not to introduce air bubbles into medium.

Incubate at 32° C for 48 hr and record the number of tubes showing gas. Gas formation should start in 24 hr.

Report results as the estimated number of bacteria per gm, the number being the reciprocal of the highest dilution in which the majority of tubes show gas formation.

INTERPRETATION OF RESULTS

The methods described are designed to give results that will serve as guidelines in helping the laboratory worker measure the microbiological and sanitary quality of raw materials during processing and in

finished products. Results obtained should prove helpful, not only in making microbiological surveys, but also to assist in finding the source of microbial contamination and locating troubles in the handling, manufacturing, and processing of cereal products.

Only experience will provide information as to the allowable limits of microbiological counts for specific products and under specific conditions. The baker or miller may be faced with problems involving "rope-producing" bacteria [3, 4] or difficulty in controlling a mold problem, while these might have only minor interest for other users of cereal products. Abnormally high microbial populations of any type of organism should be interpreted as representing poor raw material or poor methods of handling. An exact interpretation of specific numbers can be made only in the light of full information regarding the product under study and its intended use. Liberal use of the references presented should be made for the particular products under study. For example, see Jensen [19] for material on cereals in meat products, for information on flour see references 4–7, and 15, and for material on canned products see numbers 1, 2, 13, etc.

As the successive "breaks" of grain are incorporated into the flour stream the higher the counts become. Thus whole wheat flour would be expected to have higher counts than white flour. Manufactured products, such as starch would be expected to have lower counts than raw or milled products. If there is interest in specific standards for starch to be used in canning, see reference 19.

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CHAPTER VII

DEHYDRATED FRUITS AND VEGETABLES

This field has received less attention than other segments of the processed foods industry and it appears that standardized methods for the microbiological examination of these products are desirable. Methods for the examination of dehydrated fruits and vegetables are limited because, with few exceptions, the published investigations have been stimulated by wartime production of these foods.

Several studies on a significant number of samples of various dehydrated fruits and vegetables have shown very few, if any, of them to be sterile. Very little information is available concerning the significance of microbial populations found in these products. Therefore, generalizations should not be made unless the complete production and storage history is known and the analyst is an experienced observer and familiar with the technology of fruit and vegetable dehydration.

Microbiological examination can serve as a means of determining the sanitary quality, detecting unsanitary practices, and for recommending improvements in plant and handling conditions. The presence of large populations of microorganisms may be an indication of poor raw material, improper processing technics or poor plant sanitation. Microbiological information is especially helpful when the dehydrated products are to be rehydrated under conditions which would promote rapid growth, or where the reconstituted material is to be an ingredient of some prepared food product. It is to fill these needs that the following methods are recommended.

SAMPLING

Sample containers should be screw-top glass jars, or cans with tight-fitting lids, and prior to use should be cleaned, rinsed in distilled water, dried, and sterilized. They should be of a size to accommodate a liberal sampling of bulky material. Sterile paper bags may be used when the product can be handled quickly and analysis made promptly.

If the dehydrated product is in small packages a representative number should be taken and transported to the laboratory intact. If the product is in bulk or large containers care should be taken to secure representative samples by taking a portion from all sections of the containers or from the lot. At least a 16-oz sample should be taken under aseptic conditions from each lot or large container of product under examination. Following selection, samples should be handled in such a manner that no contamination or other changes occur prior to examination, and they should be examined as soon as possible after taking.

Bulky products should be crushed or ground in a sterile container so as to reduce them to a particle size suitable for weighing and to facilitate placing the bacteria in suspension in the dilution blank.

Composites of lots should be taken in preference to "grab" samples. Under conditions where very large lots are to be examined several composite samples should be taken and mixed together in preparing the sample for analysis.

DEHYDRATED FRUITS

I. Preparation of Sample

For apples, apricots, peaches, etc., select 20 pieces of fruit and aseptically weigh into a sterile screw-cap jar and add 200 ml of sterile water. Allow jar to stand 30 min at room temperature, then shake by hand or by machine for 1–3 min.

For prunes,[1] figs,[2] and dates select 10 fruits and cut in two with a sterile knife. Weigh into a sterile screw-cap jar and add 100 ml of sterile water. Allow to stand for 30 min at room temperature and then shake by hand or by machine for 1–3 min.[2]

For raisins, currants, cherries, etc., weigh approximately 100 pieces of fruit into a sterile screw-cap jar and add 200 ml of sterile water. Allow to stand 30 min and treat as above.

II. Microbiological Examination

Prepare serial dilutions from above samples using plain dilution blanks (23A).

III. Total Count

Place 1-ml aliquots in Petri plates, in duplicate, and pour with total plate count (64), or orange serum agar (47) adjusted to pH 7.0.

Incubate at 32° C for 3 days and report as total count per gm of fruit.

IV. Yeasts and Molds

Place 1-ml aliquots in Petri plates, in duplicate, and pour with malt agar (36) or potato dextrose agar, acidified (51).

Incubate 5 days at 32° C. Observe plates at 3 days in order to count plates if molds are likely to overgrow plates.

Report as yeasts or molds per gm of sample.

Alternate Method

If only a yeast count is desired add 0.25 per cent of sodium propionate to above agars to inhibit growth of mold.[3]

DEHYDRATED VEGETABLES

I. Preparation of Sample

A. *Leafy Vegetables*

Weigh 10–20 gm into a sterile bottle or flask of at least 250-ml capacity. Add 190 ml of sterile water. Allow to rehydrate for 30 min at refrigerator temperature and then shake vigorously for 2 min.

Prepare serial dilutions using plain dilution blanks (23A).

B. *Root and Solid Vegetables*

Weigh 10–20 gm into a sterile bottle or flask and add 190 ml of sterile water. Allow to rehydrate 30 min at refrigerator temperature and then shake vigorously for 2–3 min.

II. Microbiological Examination

A. *Total Count*

Transfer 1-ml aliquots to Petri plates, in duplicate, and pour with total plate count (64), or orange serum agar (47) adjusted to pH 7.0.

Incubate at 32° C for 3 days and report as total count per gm of product.

B. *Lactic-Acid Bacteria*

Transfer 1-ml aliquots to Petri plates, in duplicate, and pour with liver infusion—sorbic acid agar [4] (35), orange serum agar (47) adjusted to pH 7.0, or with Rogosa, Mitchell, and Wiseman's medium [5] (53).

Incubate at 32° C for 3 days and count typical colonies.

If confirmation of colonies is desired make Gram stains (B3), and test each colony for catalase production.

To determine presence or absence of catalase flood plates with a 3–5 per cent solution of hydrogen peroxide or test material from each colony with the peroxide on a glass slide. Gram-positive, catalase-negative, nonspore-forming rods and cocci are reported as lactic-acid bacteria.

Alternate Method

Pour media into Petri plates and when agar has solidified transfer 0.1 ml of appropriate dilutions to the center of the plate.

Spread evenly over the surface with a sterile bent glass rod.

Incubate as indicated above. If desired the plates may be incubated in an anaerobic jar with an atmosphere of nitrogen containing 5–10 per cent carbon dioxide.

C. *Spore-Forming Bacteria, Thermophilic*

1. *Preparation of Sample to Destroy Vegetative Cells*

This may be accomplished by:

a. Transfer 60–70 ml of the liquid phase of the initial rehydrated samples into a sterile tube or flask and heat to boiling for 5 min. Prepare serial dilutions using plain dilution blanks (23A).

Or:

b. Transfer 60–70 ml of the liquid phase into a sterile flask and autoclave for 10 min at 5 lb pressure. Prepare serial dilutions.

Or:

c. Pipette serial dilutions directly into melted and cooled media for the heating process as given in 1a or b.

2. *Flat-Sour Spores*

Place 1-ml aliquots from the heated dilutions (1a or b) in Petri plates, in duplicate, and pour with dextrose tryptone agar (20) to which bromcresol purple indicator (C5) has been added.

If the technic of heating in the medium is employed (1c above) pipette 1 ml of unheated dilution into 10 ml of dextrose tryptone agar (20) containing bromcresol purple (C5) and autoclave for 10 min at.5 lb pressure.

Cool to 45°–50° C quickly and pour into Petri plates. If spreaders are a problem overpour with plain agar (49).

Incubate 48 hr at 55° C and count colonies having a yellow halo. Report as numbers of flat-sour spores per gm of product.

3. *Anaerobes Producing H_2S*

Place 1-ml aliquots from heated dilutions into duplicate tubes of melted and cooled sulfite agar (59). Mix inoculum by rolling the tube between the palms of the hands until agar starts to solidify. Cover agar with sterile "vaspar" (71), or petrolatum.

Incubate at 55° C for 48 hr. Count the black colonies and report as numbers of H_2S producing anaerobes.

If dilutions are heated in the medium (1c above), cool tubes after heating and cover with "vaspar" (71), or petrolatum.

4. *Anaerobes Not Producing H_2S*

Place 1-ml aliquots of heated dilutions into tubes of heated and cooled liver broth medium (32).

Mix gently and cover surface with ½ in. of melted sterile "vaspar" (71) or petrolatum.

Incubate 48 hr at 55° C. Report anaerobes as present in the highest positive dilution.

If there is need to estimate the population of these anaerobes increase the number of tubes in each dilution to 5 and refer to Table 1, Chapter XVI to calculate the most probable numbers.

DIRECT MICROSCOPIC EXAMINATION

Allow initial rehydration jar to stand to allow suspended material to settle. Filter a portion of the liquid phase through a light cotton layer to remove coarse material.

With a Breed pipette [6] transfer 0.01 ml of liquid to a microscope slide and spread evenly over a 1-sq cm area. Dry and fix with heat or with methyl alcohol.

Stain with Gray's double dye stain (B5), or North's aniline oil methylene blue stain (B8). Rinse, dry, and examine under the microscope. Report as estimated numbers of microoganisms observed per gm of product.

This method has certain limitations and considerable judgment must be exercised in making the estimate of numbers due to fruit or vegetable material which may be on the slide and interfere with making the count of organisms in the microscope fields. Its greatest use is in observing the general extent of the microbial population and the various bacterial types present.

INTERPRETATION OF RESULTS

Abnormally high bacterial, yeast or mold counts may be indicative of poor sanitary history in either the handling of raw material or during the dehydration process. Poorly engineered drying equipment or faulty drying procedures may lead to slow drying in certain areas with development of high populations of microorganisms. Usually the temperature in dehydration equipment is high enough to deter development of such populations, but where temperatures are not uniform,

or not adequately controlled, certain areas may exist where bacteria may thrive and act as an inoculum for the entire output. Poor condition of raw material and poor handling also encourage microbial growth.

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CHAPTER VIII

EGGS AND EGG PRODUCTS

The use of eggs in many compounded food products, the effect of sanitary practices upon the microbiological picture, and the public health significance of certain types of bacteria often found in eggs lead to an interest in methods for the microbiological examination of this product. For the most part eggs are sterile at the time of laying.[1] Through contact with wet and dirty surfaces, improper handling in the channels of commerce, and through insanitary breaking plant practices some egg products may pick up a varied flora and develop enormous bacterial populations. Standardized methods are desirable in order to determine the extent and type of contamination and to use as a guide in the practice of food plant sanitation.

For convenience in presenting such methods eggs have been divided into the following:

(a) Shell eggs
(b) Liquid eggs
(c) Frozen eggs
(d) Dried eggs

SHELL EGGS

I. Sampling

Select a number of shell eggs at random from several cases or cartons. Due to lack of uniformity in the bacterial picture from one egg to another a liberal sample should be chosen and representative of the lot. Transfer the eggs to clean cartons or cases for transport to the laboratory and maintain the eggs at temperatures below 50° F during transport and until analysis is made. Avoid sweating of the eggs if at all possible. This results from transferring shell eggs from cold storage temperatures of around 30° F to room temperature without a tempering period. Under certain circumstances sweating facilitates the penetration of bacteria found on the shell into the egg meat.

II. Preparation of Sample

Wash each egg with a brush using soap and water, drain and immerse in 70 per cent alcohol for 10 min. Remove from alcohol, drain, and flame. Grasp egg by blunt or air cell end and puncture a 0.5 in. hole in the opposite end with sterile scalpel or forceps. Flame opening before removal of contents. Invert opened egg over a sterile crucible triangle placed on a tripod. Place sterile wide-mouth bottle beneath egg and force the contents out by gently applying heat with a Bunsen flame to the air cell end. One egg per container or several can be collected depending upon the purpose of the examination.

If a check of the commercial quality for plant operating purposes is all that is desired the aseptic precautions can be reduced with adequate care taken in removing the egg meat from the washed, treated shell by the use of a clean breaking knife.

Shake the contents of the sample container or beat with a sterile spoon or with a sterile electric mixer until the sample is homogeneous.

If separate examination of egg white and egg yolk is desired, they may be separated by use of a sterile commercial separator or by the use of a sterile spoon. The yolk can be freed of excess egg white by rolling it on a sterile towel.

III. Microbiological Examination

Weigh 11 gm of the homogeneous sample into a 99-ml saline dilution blank with glass beads (23F). Place stoppered dilution bottle on a shaking machine or shake 25 times by hand through a 1-ft distance. Prepare appropriate serial dilutions using saline dilution blanks (23E).

A. *Total Count*

Pipette 1 ml from the dilution blanks, in duplicate, into Petri plates and pour with total plate count agar (64). As a rule dilutions of 1:100, 1:1,000, and 1:10,000 will be sufficient. Place dilutions in only a few sets of plates at a time and pour with agar as quickly as possible. Egg products are adhesive and have a tendency to adhere to the glass of the dish if allowed to stand, with a resulting heavy grouping of colonies on the glass at the spot of inoculation and a lesser number in the agar.

Incubate at 32° C for 3 days and report the average of the 2 plates multiplied by the reciprocal of the dilution as the total number of colonies per gm of product.

B. *Yeast and Mold*

Pipette 1 ml from serial dilutions, in duplicate, into Petri plates and pour with potato dextrose agar, acidified (51), or malt agar (36). Incubate for 3 days at 32° C. Observe the plates at 1 and 2 days so that counts may be made if molds overgrow the plates. Report average number of colonies as yeasts and molds per gm of egg material.

LIQUID EGGS

(Whole egg, egg white, egg yolk, salted yolk, sugared yolk, blends.)

I. Sampling

Obtain sample material from vats or tanks in the liquid egg handling plant or from containers as delivered to the consumer. Make sure that the product has been thoroughly mixed by mechanical agitation or by hand mixing in the container. Use sterilized dipper or sampling tube to withdraw the sample. Sterile pint mason jars or friction top cans are satisfactory for holding the sample. Obtain about ¾ pt of sample and hold at a temperature below 40° F but avoid freezing as this will alter the microbiological picture. The sample should be analyzed within 4 hr after taking. If a longer delay is anticipated, use dry ice in an insulated box but avoid freezing the sample.

Record the temperature of the containers from which the samples were taken as that is often the key to abnormal bacterial populations. Temperatures above 45° F indicate improper handling of the liquid and are likely to lead to rapid deterioration.

II. Preparation of Sample

Thoroughly mix the contents of the sample jar until it is free of any lumps and is homogeneous. If sample is egg white it should be free of lumps of thick white. Care should be taken in the mixing to avoid excessive foaming of the sample.

III. Microbiological Examination

Weigh 11 gm of the sample aseptically into a 99-ml saline dilution blank with glass beads (23F). Place stoppered bottle on a shaking machine or shake 25 times by hand. Prepare serial dilutions using saline dilution blanks.

A. *Total Counts*

Pipette 1 ml from the dilution blanks, in duplicate, into Petri plates and pour with total plate count agar (64). Inoculate and pour only a few plates at a time for reasons indicated under shell eggs. For whole egg products 1:100, 1:1,000 and 1:10,000 dilutions will be usually sufficient. If the history of the samples indicates higher counts dilutions up to 1:1,000,000 may be called for. Egg white and egg yolk products generally have lower counts than whole egg. If whole egg and yolk products have been pasteurized the 1:100 and 1:1,000 dilutions may be sufficient.

Incubate at 32° C for 3 days and report the average of the 2 plates multiplied by the dilution as the total number of colonies per gm of product.

B. *Yeast and Mold*

Pipette 1 ml from appropriate dilutions into Petri plates, in duplicate, and pour with potato dextrose agar, acidified (51), or with malt agar (36). Incubate for 3 days at 32° C. Observe the plates at 1 and 2 days so that counts may be made if molds overgrow the plates. Report average number of colonies as yeasts and molds per gm of egg material.

FROZEN EGGS

(Whole egg, whites, yolks, salted yolks, sugared yolks, blends)

I. Sampling

Open the cans of frozen eggs in such a way as to prevent dirt or extraneous matter from entering the can. Remove frozen material from 3 areas of the top of the ice by means of a sterilized chisel, hatchet or other instrument. Drill 3 cores, preferably with a high-speed electric drill, from top to bottom of the container, one core in the center, a sec-

ond midway between the center and the periphery and a third near the edge of the container. Transfer the drillings to a chilled sample container with a sterile spoon. Drill a fourth hole for examination of condition of the product. Heat produced by the electric drill intensifies the odor of egg material. Immediately upon completion of the fourth hole smell the opening and the drillings and record odor as normal, stale, sour, putrid, musty, etc. The odor is often the key to the general quality of the product and the handling that it has had. Products having a normal odor as a rule have low bacterial counts.

Keep the drillings solidly frozen at all times. Pack in an insulated box with dry ice for transport to the laboratory.

II. Preparation of Sample

Thaw the contents of the sample container as rapidly as possible. Use of running tap water is recommended with bottle or jar two-thirds submerged. Rotate the container frequently until thawed. Open the container using aseptic precautions and thoroughly mix the contents to get uniformity. An alternate method is to thaw overnight in a refrigerator. Do not test samples which have arrived at the laboratory in a thawed condition.

III. Microbiological Examination

Weigh 11 gm of sample into a 99-ml saline dilution blank with glass beads (23F). Place stoppered bottle on a shaking machine or shake 25 times by hand through a 1-ft distance. Prepare serial dilutions using saline dilution blanks (23E).

A. *Total Counts*

Pipette 1 ml from appropriate dilutions, in duplicate, into Petri plates and pour with total plate count agar (64). Inoculate and pour only a few plates at a time for reasons given under shell eggs. As a rule dilutions of 1:100, 1:1,000, and 1:10,000 are sufficient, higher counts being expected in frozen whole egg products than in either egg white or egg yolk. Viable bacterial populations decrease during frozen storage and lower counts are to be expected in products which have been in storage several months. If the odor of the thawed sample is normal, the count is likely to be low, but if the odor is fruity, sour, or putrid dilutions up to 1:1,000,000 should be plated.

Incubate at 32° C for 3 days and report the average count multiplied by the dilution as the total number of colonies per gm of product.

B. *Yeast and Mold*

Pipette 1 ml from the dilution blanks, in duplicate, into Petri plates and pour with potato dextrose agar, acidified (51), or with malt agar (36). Incubate at 32° C for 3 days. Observe at 1 and 2 days so that counts may be made if molds overgrow the plates. Report average count as colonies of yeasts and molds per gm of product.

C. *Putrefactive Anaerobes*

Pipette 1 ml from serial dilutions, in duplicate, to tubes of Holman's alkaline meat medium (27). Immediately before use heat the tubes in flowing steam for 20 min, to exhaust the oxygen, and quickly cool below 37° C before inoculating.

Incubate at 37° C for 3 days and record the tubes showing gas and digestion of meat. Confirm identity of types by use of Gram stain (B3). Report the reciprocal of the highest dilution showing positive anaerobic growth as the estimated number of putrefactive anaerobes per gm of egg.

DRIED EGGS

I. Sampling

Take samples that are representative of the lot. If in bulk containers whole egg and yolk may be sampled with a sterilized trier, taking at least 3 cores to the bottom of the container. Powdered or spray-dried albumen may be taken with a trier by tilting the container. Flake or lump albumen must be sampled by means of a sterilized spoon or scoop. Resterilize equipment before removing cores from the next container.

Discard the bottom 1 in. of the egg in the trier and transfer the rest of the core to a sterile jar or friction-top can. Fill the can only ⅔ full in order to leave room for mixing.

If the product is in small packages select several representatives of the lot and take to the laboratory in the original container. Open the packages under aseptic conditions and transfer a liberal quantity to a sterile can or beaker. Thoroughly mix with a sterilized spoon to obtain a homogeneous mixture.

Samples of dried egg products should be examined as soon as convenient and should be held under refrigeration, but under conditions that will not allow absorption of moisture.

II. Preparation of Sample

Remove sample containers of whole egg and yolk from refrigeration and allow them to come to room temperature. Better dispersion in the dilution blanks will be obtained if the powder is warm. Open sample containers under aseptic conditions and thoroughly mix with a sterile spoon. If the pieces of flake albumen are thick and of large size crush in a sterilized mortar until pieces are smaller than ¼ in.

III. Microbiological Examination

Weigh 11 gm of product into a 99-ml saline dilution blank with glass beads (23F). Place stoppered bottle on a shaking machine and shake until there is no evidence of lumps. If lumps persist crush the lumps against the side of the bottle with a sterile spatula and return to shaking machine. If shaken by hand continue shaking and crushing of lumps until none are evident.

Flake or lump albumen dissolves slowly. Shake dilution blank thoroughly and place in a cool place. Shake once or twice every few min until the flakes have dissolved.

Prepare serial dilutions by transferring 1 ml of the 1:10 dilution to a 99-ml saline dilution blank (23E) taking care to shake the initial dilution before making the transfer. Follow this procedure in making other dilutions.

A. *Total Counts*

Pipette 1 ml from the appropriate dilutions, in duplicate, into Petri plates and pour with total plate count agar (64). Inoculate and pour only a few plates at a time for reasons given under shell eggs.

Incubate at 32° C for 3 days and report the average count multiplied by the dilution as the total number of colonies per gm of product.

As a rule 1:100, 1:1,000, and 1:10,000 dilutions are sufficient. However, dried egg products vary widely in viable count due to age, method

of manufacture, and type of raw product used. Storage causes a decrease in population, while the fermentation process used in albumen brings about large microbial populations. Therefore, dilutions must be chosen largely on the basis of experience and known history of the samples.

B. *Yeast and Mold*

Pipette 1 ml from the dilution blanks, in duplicate, into Petri plates and pour with potato dextrose agar, acidified (51), or with malt agar (36). Incubate at 32° C for 3 days. Observe at 1 and 2 days so that counts may be made if molds overgrow the plates. Report average count as yeast and mold colonies per gm of product.

C. *Putrefactive Anaerobes*

Pipette 1 ml from serial dilutions, in duplicate, to tubes of Holman's alkaline meat medium (27). Immediately before use heat the tubes in flowing steam for 20 min, to exhaust the oxygen, and quickly cool below 37° C before inoculating.

Incubate at 37° C for 3 days and record the tubes showing gas and digestion of meat. Confirm identity of types by use of Gram stain (B3). Report the reciprocal of the highest dilution showing positive anaerobic growth as the estimated number of putrefactive anaerobes per gm of egg.

DIRECT MICROSCOPIC COUNTS

I. Liquid and Frozen Eggs

Place 0.01 ml of undiluted egg material on a clean dry microscope slide using a Breed pipette.[2] Spread the egg material over an area of 1 sq cm. A circular area with diameter of 1.13 cm is preferred. Permit smear to dry on a level surface at 35°–40° C. Immerse in xylene for a minimum of 1 min, remove and dry and then immerse in 95 per cent alcohol for 1 min. Remove and dry in air. Stain smear for about 1 min with North's aniline oil methylene blue stain [3] (B8). Wash stained preparation by repeated immersions in a beaker of water. Dry thoroughly before examination.

Examine under the microscope using oil immersion lens. Count number of microorganisms observed in 100 fields and multiply the

average number per field by the microscope factor. Report results as estimated number of bacteria (or clumps) per gm of egg material.

II. Dried Egg Products

Place 0.01 ml of the 1:10 or 1:100 dilution of dried egg material on a clean dry microscope slide with a Breed pipette.[2] Spread the material over a 2-sq cm area. A circular area of 1.6 cm in diameter is preferred.

Lithium hydroxide dilution blanks (23C), may be used for whole egg and yolk products to obtain more complete solution of the product. If there is difficulty in spreading the smear the addition of a drop of water will be helpful.

Proceed with treatment, staining and counting as given above under liquid and frozen eggs.

Multiply the average number of bacteria observed per field by double the microscope factor, due to the use of a 2-sq cm area, and then by the dilution used to make the smear. Report the figure as estimated number of bacteria (or clumps) per gm of egg material.

INTERPRETATION OF RESULTS

High bacterial counts are undesirable in all egg products. Bacteria grow rapidly in broken-out egg products and high bacterial counts will result if the temperature is allowed to rise above 60° F or the egg liquid is held for several hours. In sanitary or plant control operations bacterial counts may be used to judge the care used in handling the product.

Low or undergrade eggs generally have high counts in the shell and when broken out will give high initial counts.[4] Also, eggs with high initial count must be handled with particular care to prevent the development of enormous populations during plant handling, freezing, or drying.

Poor sanitation in the plant, especially the poor cleaning of holding tanks, pipe lines, pumps, etc., give rise to rapid bacterial increases.[4] Improper washing of dirty eggs and the attempted salvage of very doubtful eggs contribute to high bacterial counts.

Freezing storage or storage of dried eggs brings about marked decreases in numbers of viable bacteria. Therefore, a relatively low count in these products does not necessarily indicate high quality. Something regarding the storage history of the product should also be known. Pasteurization in the liquid state will markedly lower the counts normally expected in all of the untreated products.[5]

In unpasteurized products the presence of putrefactive anaerobes, molds, and aerobic spore formers often indicates the use of shell eggs that have been in storage for some time. The presence of abnormal numbers of *Pseudomonas* types may indicate the use of washed eggs or current receipt eggs produced during a wet season. As a rule a musty odor is also encountered.

Dried egg albumen produced by the "natural" fermentation process will show higher numbers of coliform types than usual.[6] Bad odor in the albumen is an indication of an uncontrolled or faulty fermentation. *Serratia* and *Achromobacter* types often give rise to off-odors in fermenting albumen.[6]

Direct microscopic counts are indicative of the type of raw material used and the sanitary handling, as the microscope reveals the dead as well as the viable organisms. Abnormal microscopic counts are indicative of poor quality regardless of the viable count. Stuart, et al.,[7] and Hirschmann, et al.,[8] have shown that dried eggs with high counts and poor sanitary history deteriorate faster than normal. In making direct counts it is often of benefit to note the type of cell that is most dominant. Mold hyphae and large bacillus cells are indicative of use of shell eggs that have been held, while the appearance of many small short diplocells with pointed ends indicate poor sanitation and handling conditions in the plant.

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CHAPTER IX

FERMENTED FOODS

There are a number of different types of fermented foods which might be considered under this heading. The methods to be discussed in this section do not deal with dry products which have undergone fermentation during preparation, such as coffee, tea, cocoa, etc., nor with fermented beverages such as beers, wines, etc. Rather they apply to fermented products, such as sauerkraut and olives, either as raw or as processed products.

SAUERKRAUT

Sauerkraut is normally sold as a processed food or as a raw product. It may be abnormal in several ways, such as poor color, softness, or low in acidity. These may often lead to an abnormal microbial flora. Usually such conditions are caused by faulty salting, improper temperature of fermentation, or poor covering of vats. These conditions may result in improper acid development or a destruction of acid. With this in mind it is essential that after making a preliminary examination of the food an analysis for total acidity and salt should be made.

The total plate count has little significance in indicating the condition of raw kraut. It is used primarily for isolation of organisms from the kraut and to study types of organisms developing in the product.

For raw material obtain samples of juice representative of the lot, especially with reference to both top and bottom of the containers. For processed products take cans representative of the lot and withdraw juice in an aseptic manner. Loss of vacuum or development of pressure in the cans is indicative of microbial growth or of nonbacterial hydrogen production.

I. Microbiological Examination

A. *Total Count*

Weigh 11 gm of sample into a 99-ml sterile water blank (23A). Prepare dilutions in the customary manner. For raw products transfer

1-ml aliquots to Petri plates, in duplicate, from dilutions of 1:10,000 to 1:1,000,000. For processed products make platings of original juice and 1:10 dilution.

Pour plates with total plate count agar (64) and incubate at 32° C for 48–72 hr. Report as total numbers per ml of juice, or as numbers of certain characteristic colonies per ml.

B. *Yeasts and Molds*

1. Transfer appropriate dilutions to Petri plates, in duplicate, and pour with potato dextrose agar, acidified (51), malt agar (36), or dextrose agar, acidified (17).

2. Incubate at 32° C for 5 days. Observe plates at 3 days in order to make counts if plates are likely to be overgrown.

3. Report as numbers of yeasts or of molds per gm of product.

C. *Acid-Forming Organisms*

1. Transfer appropriate dilutions to Petri plates, in duplicate, and pour with APT agar (5), total plate count agar (64) to which brom-cresol purple (C5) has been added as an indicator, or liver infusion-sorbic acid agar (35).

2. Incubate at 32° C for 72 hr and count the colonies showing a strong acid reaction. Report as numbers of acid formers per gm of product.

3. If confirmation as to type of organism is desired make a Gram stain (B3) and test each colony for catalase production.

4. When the smears have been made, flood surface of the plates with a 3–5 per cent solution of hydrogen peroxide and observe with a hand lens or a wide field binocular. Those colonies which exhibit no evidence of gas formation are catalase-negative. This may also be done by transferring material from a colony to a microscope slide and mixing with the peroxide solution. Report as lactic acid-forming bacteria those colonies that are catalase-negative, Gram-positive, nonspore-forming rods and cocci.

II. Interpretation of Results

Raw products normally contain large numbers of organisms, the colonies being small, gray-white, round, 1–3 mm in size, or lens-shaped subsurface colonies of that size and larger 3 mm surface colonies. The most frequently encountered organisms are *Leuconostoc mesenteroides*, *Lactobacillus plantarum*, and *Lactobacillus brevis*.[1] Salt concentrations of around 2.2 per cent influence normal fermentations. Lower salt contents tend to produce softer sauerkraut while higher concentrations inhibit the normal bacterial flora and reduce the general quality.[2]

The presence of large white or cream-colored colonies or small colored colonies are indicative of an abnormal product. The more common undesirable microbial changes in this product are due to yeasts.[3]

Processed sauerkraut should contain few microorganisms. The organisms in completely fermented kraut are killed at relatively low temperatures. Only about 140° F for 3–4 min are required to kill most of the organisms.[4] If too little processing has been given or recontamination has taken place, presence of spoilage organisms will be demonstrated by the plating technic. The presence of lactic acid formers or yeast colonies in a processed product normally indicates underprocessing or growth after processing.

III. Direct Microscopic Examination

Since excessive numbers of yeast cells, mold hypha, or Gram-negative rods are indicative of an abnormal condition, a microscopic examination of a Gram-stained preparation is desirable.

Thoroughly mix juice from the sample and using a Breed pipette[5] spread 0.01 ml over a 1-sq cm area on a microscope slide. After drying fix preparation with heat and stain with Gram's stain (B3). Observe under microscope and make differential count of microbial types.

IV. Interpretation of Microscopic Observation

A high count of Gram-positive rods and cocci should be expected and is desirable in a normal product, since they are the bacteria responsible for normal fermentation. On the other hand, a high yeast count or presence of mold hypha are indications of faulty technics, such as excessive salting, exposure to contamination from the air, or drying of the surface. Very small Gram-negative rods of the coliform type should not be present.

OLIVES

The major portion of the olives grown in California undergo a fermentation before subsequent processing. Those imported into the United States are shipped in brine after undergoing fermentation. Olives undergo a "natural" fermentation in which lactic acid is produced. If conditions exist which alter this desirable fermentation, spoilage may result. This section deals with methods recommended for the study of organisms found in normal olives and for those types that cause spoilage and deterioration.

Collection of Samples

Most of the organisms found in olives are found in the brine. Collect samples of brine aseptically and representative of the container. For barreled olives this can be done with a pipette of large bore and the brine transferred to a sterile bottle. If the olives are in jars or small containers, they should be moved to the laboratory where examination can be made more conveniently.

Where the defect is found in the olive itself, such as yeast spots, individual fruits should be selected and placed in a sterile jar.

I. Microbiological Examination

Weigh 11 gm of brine into a 99-ml water blank (23E). Prepare other dilutions as experience dictates.

A. *Lactic Acid Bacteria*

Prepare Petri plates of liver infusion-sorbic acid agar (35), orange serum agar (47), or Rogosa, Mitchell and Wiseman's medium (53). Pour medium into plates and allow to solidify.

Transfer 0.1-ml portions of appropriate dilutions to surface of agar in center of the plate and spread evenly over entire area with a sterile bent glass rod.

Incubate aerobically at 32° C for 3 days or if possible incubate in an anaerobic jar under nitrogen atmosphere to which 5–10 per cent carbon dioxide has been added.

Count the colonies and select representative ones for the preparation of smears for staining and for determination of catalase production according to method under Sauerkraut C, 4. Report as numbers of acid formers per gm of product.

Alternate method

As an alternate, plate appropriate dilutions with APT agar (5) to which bromcresol purple indicator (C5) has been added. Incubate at 32° C for 3 days and count acid-producing colonies.

B. *Bacteria-Causing Softening*

Certain members of the genus *Bacillus* have been associated with softening in olives.[6]

Inoculate the surface of a slant of polypectate gel medium (50) with 1 ml of appropriate dilutions of brine sample.

Incubate at 32° C for 3 days and observe for liquefaction or weakening of gel in the slant. Longer incubation may show complete liquefaction. Report softening organisms as being present in the highest dilution showing softening of the gel.

Alternate method

If a more accurate estimation of numbers of these organisms is desired, inoculate 5 tubes from each dilution and after incubation determine the most probable numbers of softening organisms by referring to Table 1, Chapter XVI.

C. *Detection of Lactic Acid Bacteria in "Yeast Spots"*

One of the most common abnormalities associated with olives is known in the industry as "yeast spots." These are small white spots which develop between the skin and the flesh of the olive. Microscopic examination and culture studies have shown this defect or blemish is due to bacteria (*Lactobacillus plantarum*) rather than yeasts.[7, 8] Select olives showing "yeasts spots" and rinse in several changes of sterile water.

Immerse for 10 min in a 1.0 per cent solution of mercuric chloride. Rinse in several changes of sterile water.

Open individual pustules with a sterile dissecting needle and with an inoculating needle transfer a portion of the contents to the surface of orange serum agar (47) or liver infusion-sorbic acid agar (35) by touching in 3 separate spots. Material from the pustule should also be used for a Gram stain (B3). Incubate at 32° C for 24–48 hr.

Observe for lactic acid-producing types of bacteria and if necessary make Gram stains from the colonies and test for catalase production by method under Sauerkraut C, 4.

Alternate method

In order to obtain well isolated colonies transfer a small portion of the material in the pustule to a test tube of sterile water by means of an inoculating needle.

Shake to obtain suspension of the organisms and then streak on the dry surface of above media in such a way as to obtain isolated colonies after incubation.

If the olive samples are older than 3 months, isolations generally cannot be made due to the death of the organisms.[8] Therefore, it is necessary to know age of the olives if one is to recover viable cultures.

D. *Aerobacter Types of Bacteria*

Coliform bacteria of the genus *Aerobacter* frequently cause gas-pocket formation in the flesh of olives in brine.[7, 9] The presence of these coliform bacteria in olives is not necessarily indicative of lack of sanitation, but places emphasis on their role in abnormal fermentations.[10] To detect the presence of members of this group streak a loopful of brine on the dry surface of Levine's eosin methylene blue agar (31) so as to obtain well isolated colonies.

Incubate at 32° C for 48 hr and observe for large, pink, moist colonies typical of the aerogenes type.

To estimate numbers of these organisms transfer 1-ml aliquots of the dilutions to each of 5 tubes of lactose broth (29). Incubate at 32° C for 48 hr and record the tubes showing gas.

From the last 3 dilutions showing gas streak each positive tube to the surface of Levine's eosin methylene blue agar (31).

Incubate at 32° C for 24–48 hr and observe for typical *Aerobacter aerogenes*-type colonies.

Refer to Table 1, Chapter XVI, and report as most probable number of *Aerobacter*-type organisms.

E. Yeasts and Molds

Place 1-ml aliquots from appropriate dilutions of olive brine in Petri plates, in duplicate, and pour with malt agar (36) or potato dextrose agar, acidified (51). Incubate at 32° C for 3 days and make differential count for yeasts and molds.

If there is need to obtain only yeast colonies the molds may be inhibited by adding 0.25 per cent of sodium propionate to malt agar and incubating as usual.

F. Clostridia Associated with Zapatera Spoilage

Zapatera is a malodorous fermentation that sometimes occurs in all types of brined olives.[11] Species of the genus *Clostridium* have been associated with this spoilage which results from end products of the decomposition of lactic and acetic acids in the brines.[12] Recognition of Zapatera spoilage is largely by odor, but it is stressed that the sensitivity of individuals to this off odor varies. At first the odor is cheesy or sagey, but as spoilage progresses the odor changes and becomes more pronounced and finally takes on a fecal stench or old shoe odor. In plant control work the sampler should be tested to determine his sensitivity to such odors in order to be able to recognize them in the early stages before spoilage has progressed very far.

In order to isolate organisms causing the spoilage it is necessary to proceed through an enrichment technic.

Neutralize the acidity of suspected brine with sterile calcium carbonate.* Place 0.5 gm of sterile calcium carbonate in a sterile 25 x 200-mm tube, with a screw cap, and add 10 ml of brine.

Heat neutralized brine to 80° C and hold for 5 min and then cool quickly in crushed ice and water.

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* Sterilize in the dry state or in the form of a slurry by autoclaving for 30 min at 121° C (15 lb pressure).

Transfer 1-ml aliquots, in duplicate, to sterile 15 x 200-mm Veillon tubes [13] containing 0.5 ml of sterile 5 per cent sodium bicarbonate solution,* to hasten rapid germination.

To the tube add fresh acetate-lactate medium (1) until ¾ full, seal with melted vaspar (71) or petrolatum. Incubate at 32° C for 24–48 hr.

As soon as gas appears in enrichment medium, transfer 0.5–1 ml of the material from the Veillon tube by pipette to a fresh tube of enrichment medium as described above.

Repeat this operation until at least 4 successive enrichments have been made. This is to minimize the contamination by other types of bacteria. From the bottom of the last enrichment tube take a small amount of material and streak a loopful on the surface of a Petri plate of acetate-lactate agar (2). Cover plate with a porous top and incubate under strictly anaerobic conditions for 3–5 days at 32° C.

Make Gram stains (B3) from colonies which develop on plates and observe for clostridia. If identification of the cultures is required it is recommended that the combined methods as described by Spray,[14] Reed and Orr,[15] and McClung [16] be followed.

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CHAPTER X

FRUIT JUICES AND CONCENTRATES

The processing of fresh and frozen fruit juices and concentrates is a relatively new industry and one which requires close supervision in order to control the quality of the products. The use of citrus juices and concentrates has shown remarkable growth. Control of quality in these products is essentially a control of sanitation and cleanliness in handling the food in the plant. Closed systems should be used wherever possible, not only to prevent oxidation and loss of nutritional values, but to prevent microbiological contamination and spoilage from taking place. Time and temperature relationships of processing should be given special attention, and frequent cleaning and sterilizing of equipment is essential. Unless these precautions are taken, microorganisms may become firmly established at some point in the system and become the reservoir for continuous contamination. The following methods are recommended as useful tools in assaying for sanitary quality and in following plant processes and product handling.

SAMPLING

Obtain sample in original containers if possible or directly from the processing line and examine according to the following procedure according to type of product. From the viewpoints of method of examination and interpretation of results, these products should be considered as (a) Juice, or (b) Concentrate. Both may be raw or pasteurized, and held under room temperature, cool storage, or frozen storage, according to type of product.

Before and during the opening of the container, examine for signs of fermentation, or mold growth. Certain containers may show considerable evidence of pressure due to fermentation, or hydrogen swells may form due to the action of the acids on metals. For juices mix the contents of cans by inverting. For concentrates open container under aseptic conditions and stir several times to obtain good distribution.

Concentrates should be reconstituted to the orginal single strength by the use of sterile distilled water. In cases where special examina-

tion is to be made, such as for coliforms, the reconstitution should be made with a sterile sodium hydroxide solution of approximately 0.6 per cent to adjust to approximately pH 5.5–6.0. The resulting solution may be used directly for the direct microscopic count. Reconstitution should be made to a constant of 12° Brix or to that of single strength juice. For a 4 to 1 concentrate weigh 50 gm of concentrate into a 150-ml sterile water blank. When other fold concentrates are used the dilution should be made accordingly.

MICROBIOLOGICAL EXAMINATION

Raw juices and concentrates may have a high count of microorganisms and therefore serial dilutions from 1:100 to 1:10,000 are in order. Frozen or cool stored juices and concentrates may be considered as raw products. However, certain types of organisms die during storage, while others accustom themselves to low temperatures and the acid environment. Therefore, if the age of the product is known it will be of some assistance in determining the dilutions to use.

Pasteurized juices and concentrates should be relatively free of microorganisms and 1 ml and 0.1 ml should be plated in their examination unless there is evidence of fermentation or spoilage.

I. Total Count

1. Weigh 11 gm of juice, or reconstituted concentrate, into a 99-ml dilution blank (23A), shake thoroughly and prepare dilutions.

2. Transfer appropriate aliquots to Petri plates, in duplicate, and pour with total plate count agar (64), or orange serum agar (47). The latter is recommended for plating citrus products.

3. Incubate at 32° C for 72 hr. Count colonies and report as total numbers per gm of product.

II. Yeasts and Molds

1. Transfer appropriate aliquots to Petri plates, in duplicate, and pour with potato dextrose agar, acidified (51), malt agar (36), or with total plate count agar (64) to which 5 per cent of sterile juice or equiva-

lent sterile concentrate has been added. It is best to add the type of juice under examination.

2. Incubate at 21° C for 5 days. Examine at 3 days in order to count if spreaders are developing.

3. Report as numbers of yeasts and molds per gm of product.

III. Acid-Forming Bacteria

1. Prepare a set of Petri plates as for Yeasts and Molds and pour with APT agar (5), or total plate count agar (64) to which bromcresol purple has been added as an indicator.

2. Incubate at 32° C for 5 days. Count the colonies showing a strong acid reaction and report as acid-forming organisms per gm of product.

3. For confirmation as to type of acid former prepare a Gram stain (B3) and observe under the microscope.

4. If it is desirable to further identify colonies on the plate, after making the Gram stain, flood the plate with a solution of hydrogen peroxide and observe with a hand lens or wide field binocular. Those colonies showing gas bubbles are catalase-positive. Those having characteristics of acid formers but showing no gas bubbles are catalase-negative and may be considered lactobacilli.

DIRECT MICROSCOPIC EXAMINATION

The direct microscopic examination may be used for rapid screening as to general quality, plant control or for collecting data on the sanitary history of samples. Also, this offers a means of determining original quality of products held in storage for extended periods. There is a rapid drop in viable count during frozen storage, and after 8–10 months of storage the plate count may be no more than 10 per cent of the original. The rate of kill is dependent upon the temperature of storage as pointed out by Weiser and Osterud.[1] They found the rate of death much greater at temperatures above —20° F than below that point.

Because of the high sugar content of these products it is not possible to make smears for direct counting in the ordinary manner due to the dissolving of the film during staining and washing. The following recommended technic overcomes this difficulty.[2]

I. Single Strength Juice

1. Thoroughly mix a composite sample of single strength juice and adjust with sterile sodium hydroxide to a pH in the range of 5.5–6.0. Staining is more effective in this range.

2. To 10 ml of adjusted juice add 10 ml of Wolford's stain (B10) in a stoppered bottle or stoppered test tube. Mix thoroughly by inverting the tube 25 times. Do not shake as foaming is to be avoided.

3. With a Breed pipette [3] transfer 0.01 ml of the stain-juice mixture to a clean slide and quickly spread over a 1-sq cm area with an inoculating needle.

4. Dry slide under an infra-red lamp suspended so as to dry the slide in 3–4 min without browning. A 250-watt heat lamp supported about 1½ in. above the slide will do this.

5. Examine slide under oil immersion using a microscope with a field 0.16 mm in diameter. The microbial cells appear as dark blue bodies against a pink to purple background containing unstained fruit material.

Examine 100 fields and count cells. Multiply the number of cells observed by 10,000 to obtain the estimated number per ml of juice. In the case of concentrates multiply also by the dilution used in reconstituting the product.

II. Concentrates

1. Mix a composite sample of concentrate thoroughly and reconstitute a 20-gm portion to 12° Brix using a sodium hydroxide solution of a strength to adjust the pH in the range of 5.5–6.0. In the case of orange concentrate this is approximately 0.6–0.7 per cent, depending on the type of product. Mix thoroughly until concentrate is in solution.

2. Proceed as in I, Single Strength Juice, above.

For plant control purposes or where direct examination is used for screening samples a lesser number of fields may be observed. Where only a general quality examination is desired 25–30 fields may prove

sufficient. However, if the count is low it may be necessary to count 100 fields to obtain accuracy due to the large microscope factor.

INTERPRETATION OF RESULTS

High mold or bacterial counts may be indicative of poorly cleaned equipment, or reflective of the condition of the fruit used and high yeast or lactic acid-forming bacteria may be an indication of fermentation of the product. High yeast or lactic acid-forming bacteria content in concentrates may also result from entrainment of juice in the condenser leg of the evaporator. When counts are excessive a check of the entire process should be undertaken.

The use of a good quality raw fruit is essential to the production of a high quality product of low microbial count. Wolford and Berry [4] have shown that juices produced from lots containing soft-rot fruit contained many times the total bacterial numbers found in juice from sound fruit. Faville and Hill [5] found that strains of species of the genera *Leuconostoc*, *Aerobacter*, *Lactobacillus*, *Xanthomonas*, and *Achromobacter* may grow in raw fruit and that excessively high counts in the juice may indicate inadequate inspection and incomplete elimination of unsound fruit.

Wolford and Berry [6] studied the bacteria isolated from machinery and processing lines in citrus processing plants and found that in addition to other types of organisms *A. aerogenes* were abundant in the slime on conveyor equipment and wash tanks. Hays and Riester [7] have shown that high *Leuconostoc* and *Lactobacillus* counts may result from contamination in the evaporator in making orange concentrate. Brokaw [8] has pointed out the vital role of plant sanitation in maintaining quality in juice and concentrate production.

Counts on pasteurized juices must necessarily be low in order to insure keeping quality, but spore-forming bacteria and mold spores often survive pasteurization, especially if the products have not been handled properly. Mold or bacterial colonies indicate faulty washing of fruit and cleaning of equipment, or inadequate processing.

Frozen products are similar to raw juices, although a reduction in count should follow prolonged storage. An excessive yeast count may indicate growth of yeast during a slow freezing operation. Occasionally

mold growth or yeast growth may take place during cool storage if the temperature is allowed to rise.

Direct microscopic counting interpretations should be made on the basis of history of the sample. In stored juices and concentrates the difference between the viable count and the microscopic count is likely to be very great as pointed out above. By observing the type of cells present as well as the numbers one can often get a lead on the source of the contamination and develop a remedy for the trouble.

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CHAPTER XI

FROZEN FRUITS, VEGETABLES, AND PRECOOKED FROZEN FOODS

The processing and use of frozen food products has increased to large proportions and, with the perfection and availability of freezing and storage facilities for the home, should continue to increase.

In testing frozen fruits and vegetables for microbiological content consideration must be given to the fact that freezing storage at temperatures of approximately —10° F (—23° C) brings about a reduction in viable microorganisms. This may amount to roughly 50 per cent in 6 months or 75 per cent in 1 year. Thus, while a high bacterial plate count on frozen products may be taken as proof of poor sanitary history, a low or moderate count does not by itself prove the opposite. Something of the history must be known before a true interpretation may be made.

It is recommended that in the preparation of samples of frozen fruits and vegetables for microbiological analysis a mechanical "blender" be employed. This has been arrived at after considerable experience with various methods of preparation on a wide variety of frozen products. In comparison with washing or hand grinding the mechanical disintegration will, as a rule, yield higher bacterial counts, however, more uniform results will be obtained and the method is less laborious. Similarly, the choice of tryptone glucose yeast agar rests on demonstrated superiority of this medium over nutrient agar or glucose agar, although glucose agar as a medium for frozen peas appears to be only slightly inferior.

It has been found that for the most part methods and directions for frozen fruits and vegetables can be used for precooked frozen foods, although many of them are quite different in composition, form, and texture.

SAMPLING

From the lot to be examined select a suitable number of packages and transport in dry ice to the laboratory for analysis. Place samples in a

freezer (0° F) storage chest until they are to be analyzed. If material is in bulk containers take representative portions, place in sterile, pre-chilled containers, and pack in dry ice for transport to laboratory.

FROZEN VEGETABLES

I. Macroscopic Examination

Open the package and note condition of the product. If unusually large quantities of ice crystals are present on the inside walls of the package and the vegetables appear somewhat shriveled, it is suggestive of long-time storage. If there is a heavy layer of ice on the bottom of the package, it is suggestive of thawing and subsequent refreezing. Record observations and note any abnormalities, such as unnatural color or odor, or pink colonies of torulae which are indicative of improper handling practices.

II. Microbiological Examination

A. *Peas, Lima Beans, Cut Corn, Whole or Regular Cut Green Beans, etc.*

Total Counts

Break the sample, if not loose-frozen, into small units by tapping unopened package sharply against a table edge or by sharply striking with a dull instrument, being careful not to break open the package. Open package and remove sample with sterile spoon or knife, taking the sample from various parts of the broken-out package, e.g., from center and corners.

Weigh a 50-gm sample aseptically into a sterile mechanical blender cup. Add 450 ml sterile water and blend contents for 2 min. If a Variac transformer is available, it is advisable to increase the speed of the motor gradually and then run the blender for the required time at the 100 V setting.

Allow the sample to stand for 2–3 min to permit foam to subside. Resuspend sediment by swirling and pipette 11 ml of liquid into a

99-ml sterile water blank (23A). Shake briskly 25 times through a 1-ft arc. Make appropriate dilutions and pipette in duplicate into Petri plates. Dilutions of 1:100, 1:1,000, and 1:10,000 will usually suffice for commercially packed frozen vegetables, although further dilutions should be made if history or appearance of the sample warrants.

Pour with melted total plate count agar (64), cooled to 45° C, and thoroughly mix the dilution water with the agar by gently rotating plates in a figure 8 motion with slight tilting, or by mechanical means; harden, invert, and incubate at 32° C for 3 days.

After incubation count the plates and report average number of colonies as total plate count of microorganisms per gm.

B. *Frozen Spinach*

Open the package and weigh 50 gm of contents into a sterile mechanical blender cup. For proper comminution of spinach in a "blender" it is necessary to allow the package of spinach to defrost partially by standing at room temperature for 1½–2 hr or take small pieces of frozen material cut out with a sterile knife. Assemble the sample from various portions of package taking care to select petiole and blade portions in about the same ratio as in the sample. Add 450 ml sterile water, blend for 2 min and proceed as in analysis of A (peas, lima beans, etc.).

C. *Frozen Broccoli and Cauliflower*

Defrost partially at room temperature. Using a sterilized scalpel or scissors cut portions from the curd and stem of several representative pieces of the vegetable maintaining the relative proportions. Aseptically transfer 50 gm of these portions into the sterile mechanical blender cup, add 450 ml sterile water, and proceed as directed for A (frozen peas, etc.).

D. *Frozen Asparagus*

Partially defrost at room temperature. Cut spears into short lengths using sterilized scalpel or scissors. Transfer aseptically 50 gm of the segments into a sterile mechanical blender cup, add 450 ml sterile water, and proceed as in analysis of A (frozen peas, etc.). When weighing sample portions, a number of spears should be selected, and

the proportion of butts and tips maintained. Weigh cut-up type of asparagus directly into the blender cup.

III. Direct Microscopic Count

The direct microscopic method has an advantage over the plate count method in that it can be done more rapidly and requires less equipment and glassware. Also, it detects dead as well as live microorganisms and indicates sanitary history, irrespective of the viable count.

Weigh 50 gm of the vegetable into a mechanical blender cup. Add 100 ml of distilled water and blend briefly. Allow debris to settle and using a Breed pipette [1] transfer 0.01 ml of the washings to a microscope slide. Using a needle, spread the drop evenly over a 1-sq cm area of the slide. Dry and fix with heat or with methyl alcohol. Stain with Gray's double-dye stain [2] (B5), or with North's aniline oil methylene blue stain [3] (B8), rinse, dry, and examine under the microscope, using the oil immersion lens.

The microscope must be calibrated in order to obtain a factor for calculating the results. Use an ocular micrometer, such as a Whipple disk or Howard disk, with the microscope tube so adjusted that the graduation is equal to 0.1 mm (area of field 0.1 sq mm). If the tube cannot be adjusted determine the size of field within the circle of the disk and calculate the factor. Count the cells in 100 fields and multiply by the factor for the microscope and the dilution to bring to the gm sample basis. Report as microorganisms observed per gm of material.

IV. Interpretation of Results

Plate counts of more than 400,000 per gm of peas or direct microscopic counts of over 1,000,000 per gm may be considered indicative of poor sanitary conditions in the plant, or poor handling practice in transit, or in warehousing. In regard to other frozen vegetables it would appear that counts tend to run higher. In vegetable freezing plants plate counts of over 500,000 per gm of newly frozen produce are not encountered unless there is some degree of carelessness in the plant, such as faulty clean-up practice or prolonged holding of the material after blanching. Vaughn, et al.,[4] have shown that sanitation must be a continuous process in food-handling plants to insure low bacterial counts.

FROZEN FRUITS

I. Macroscopic Examination

Open the package and observe condition of the product. If large quantities of ice crystals are present on the inside walls of the package and the fruit is somewhat shriveled, it is suggestive of thawing and subsequent refreezing or of long-time storage. If there is considerable ice in the bottom of the package of dry-packed fruit, the product may have been thawed and refrozen.

II. Microbiological Examination

Hold the package of frozen fruit at room temperature for 1–2 hr to temper before opening. While the fruit is still partially frozen cut portions from various parts of contents of the package using a sterilized scalpel. The proportion of fruit to syrup should approximate that of the whole package. Weigh 50 gm of fruit and syrup into a sterile mechanical blender cup, add 450 ml sterile water, and blend for 2 min.

Total Counts

Make a 1:100 dilution by adding 11 ml of the supernatant liquid to a 99-ml sterile water blank (23A) and make further dilutions in the usual manner. Plate 1-ml portions in duplicate from the various dilutions, pour with total plate count agar (64), and incubate for 3 days at 32° C. Count colonies and report average results as total plate count of microorganisms per gm.

Yeasts and Molds

Place 1-ml portions of the appropriate dilutions, in duplicate, in Petri plates and pour with potato dextrose agar, acidified (51), dextrose agar, acidified (17), or malt agar (36). Incubate for 5 days at 21° C. Observe the plates at 3 days in order to make counts if molds are overgrowing the plates. Report average counts as numbers of yeast or mold colonies per gm of product.

III. Direct Microscopic Counts

Make direct microscopic counts according to method given under Frozen Vegetables and for yeasts, molds, and fragment counts accord-

ing to the method given in Official Methods of Analysis, AOAC,[5] for microscopic analysis of tomato products. Care must be taken to distinguish between mold hyphae and fruit setae.

IV. Interpretation of Results

Sound fruit should not show more than 20 per cent positive fields using the Howard counting method.[3] If the pack has a poor sanitary history it will show up in the high count of molds and yeasts observed under the microscope.

EXAMINATION OF PRECOOKED FROZEN FOODS

This field is a broad one in that a wide variety of food products may be prepared and offered to the public. Many of these foods result from the compounding of several previously cooked and processed items into a single product, which is not further cooked before being frozen.[6, 7] Others are compounded and subjected to cooking or sterilizing temperatures in sealed containers, after which they are frozen. Still others are cooked or baked by the consumer after defrosting.

If properly handled, the bacterial counts of precooked frozen food should be low. However, any carelessness in handling or lack of good sanitary practices may allow the development of enormous bacterial populations in such foods before they are frozen. Such counts may result also from high-count raw products, extensive handling during preparation, or improper delays encountered before freezing.[8, 9]

Since precooked frozen foods are often only warmed to serving temperature by the user, and thus not subjected to sterilizing temperatures, there is need for good sanitation, rapid handling, quick cooling and freezing, and other good plant practices. Also, frequent plant checks for possible sources of contamination should be part of the plant sanitary and quality control program, and the survey program of public health officials.

Precooked frozen meals are made up of several components which are prepared separately and placed in a common or compartmented container. In order to obtain adequate microbiological information it is necessary to examine each of the components. The entire portion of the component should be weighed and placed in the sterile blender cup,

or a representative weight taken from the portion if it is large. If the portion weighs more than 50 gm, a 1:5 or 1:2 suspension may be prepared instead of the customary 1:10, which would be beyond the capacity of the blender cup. The 1:10 can then be prepared by dilution. Experience has shown that the Frozen Fruits and Vegetable methods can be used on frozen meals without alteration, except that precooked frozen foods should not be partially thawed.

The sample should be prepared for examination as under Frozen Fruits and Vegetables, depending upon the character of the product.

I. Macroscopic Examination

Open the package and note condition of the product, including appearance and odor. Sour, putrid, or other off odor will indicate a product which has fermented during processing, by reason of a slow freeze or allowed to thaw. A dry bleached surface is indicative of improper packaging, excessive storage periods, or widely varying temperatures in frozen storage.

II. Microbiological Examination

Total Counts

Proceed according to directions given above for Frozen Fruits and Vegetables. After making appropriate dilutions pour Petri plates, in duplicate, with total plate count agar (64) and incubate for 3 days at 32° C. Report average results as total number of microorganisms per gm.

Yeasts and Molds

Proceed as for yeasts and molds under Frozen Fruits using potato dextrose agar, acidified (51), dextrose agar, acidified (17), or malt agar (36). Incubate at 21° C for 5 days, observing at 3 days for possible overgrowing of the plates by molds. Report average counts as numbers of yeast or mold colonies per gm of product.

III. Direct Microscopic Count

Weigh 50 gm of product into a sterile mechanical blender cup, add 450 ml of sterile water, and blend for 3 min. Proceed as given above for Frozen Vegetables.

IV. Interpretation of Results

Plate counts in excess of 100,000 per gm are indicative of poor handling and indicate that a check of plant operations should be made. The wide variety of products and the wide range of direct microscopic counts to be expected does not permit the establishment of reasonable limits for the count. However, it is believed that direct counts in excess of 5,000,000 per gm are an indication that checks of the plant sanitation and handling are in order. Such counts coupled with any off odor indicate products of doubtful quality.

Foods containing fat, eggs, etc., may give smears that stain poorly and considerable judgment should be used by the technician. Such slides may be defatted by immersing in xylene for 1 min prior to the staining.

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CHAPTER XII

MAYONNAISE, SALAD, AND FRENCH DRESSINGS

The widespread use of mayonnaise, salad dressings, and related products has made them important food items. As with other prepared foods there is always the possibility of contamination with resulting spoilage [1] and bacterial conditions of public health interest.[2] There is, therefore, a need for methods to detect and enumerate those microorganisms that may cause trouble. Such organisms may come from raw material, lack of proper sanitation during manufacture, or they may multiply in the final product due to formulations that are not inhibitory to their growth. At present there is a standard of identity for mayonnaise and standards exist for salad dressing and French dressing.

The following methods are recommended for the examination of this group of products.

COLLECTION OF SAMPLES

The various dressings are generally sold in glass jars or bottles containing from 6 to 32 oz per container. In this case select a number of containers representative of the lot. Gallon glass jars are commonly used for marketing larger quantities for institutions, hotels, restaurants, etc. A gal sample or an aliquot of at least 8 oz should be obtained after thoroughly stirring the contents of the gal jar with a sterile rod or spatula large enough to reach to the bottom. Transfer at least 8 oz into a sterile container, such as a bottle with glass stopper, clamp-type fruit jar, or other suitable container.

Samples collected in the unopened containers need not be refrigerated during shipment. Samples collected aseptically from opened containers should be kept below 40° F (50° C) during transportation but should not be frozen.

At the time of sampling make a record of any abnormality in appearance of the product or of off odor or flavor. Evidence of a liquid phase,

graininess, or change in color are indicative of spoilage or poor condition of the product.

Make a pH determination by diluting the sample with 5 times its volume of distilled water and using a glass electrode. The pH of salad dressings should be approximately in the range of 3.0–3.7, French dressing 3.0–4.0, and mayonnaise 3.0–4.0. If the pH is markedly above this range, spoilage may be found to result from too low an acidity.

PREPARATION OF SAMPLES

Thoroughly mix the samples using a sterile spatula. This is especially necessary due to the nature of material which in many cases is a solid emulsion. Liquid emulsions should be vigorously shaken 25 times before making dilutions.

Make the initial dilutions from each sample by weighing 20 gm of material into 10 ml of sterile water. Agitate the contents thoroughly and vigorously for 10 min either on a shaking machine or by hand. Solubilizing agents, such as Tweens, may be used, but repeated platings on different media have shown no added advantage.[1]

Make additional dilutions by weighing 11 gm from the original 2:1 dilution above, into a 99-ml dilution blank (23A). It is seldom necessary to make dilutions greater than 1:100.

MICROBIOLOGICAL EXAMINATION

I. Total Count

Distribute aliquots of appropriate dilutions into Petri plates, in duplicate, and pour with total plate count agar (64). Incubate at 32° C for 48 hr and report as total numbers of colonies per gm of material.

II. Yeasts and Molds

Place aliquots of appropriate dilutions into Petri plates, in duplicate, and pour with potato dextrose agar, acidified (51). When there is considerable gas in the dressings and yeasts are suspected as the cause, it is necessary to use a different medium.[3] In such cases use Trommer's

malt extract agar (38) containing 15 per cent of dextrose for French dressing and 30 per cent dextrose for mayonnaise.[1, 3] Potato dextrose agar, acidified (51) or malt-dextrose agar (37) may be used, but fewer yeasts will grow and a longer time is required for their growth. Incubate for 5 days at 32° C. Count at 3 days if there is danger of molds overgrowing the plates. Make a differential count and report numbers of yeasts and molds per gm of product.

III. Lipolytic Bacteria

The presence of lipolytic bacteria may be determined by the use of Starr's spirit blue agar (58).[4] Pipette aliquots of dilutions into Petri plates, in duplicate, and thoroughly mix with the above medium. Incubate 5 days at 32° C and report as number of lipolytic bacteria per gm of product.

INTERPRETATION OF RESULTS

The high acid content of mayonnaise, salad dressing, and closely related products suggests the improbability, but not the impossibility, of spoilage in these products and of the survival of organisms of public health significance. If there is insufficient acidity or an abnormal degree of contamination there is likelihood of trouble. Most aerobic and sporogenic bacteria soon die in this type of product.

From an economic aspect the problem is one of yeast spoilage. Unless proper sanitary precautions are taken, there is danger of yeast spoilage by those forms which are not inhibited by the acidities used. There is also the possibility of lactobacilli causing trouble in this type of product, since the acidity is not inimical to their growth.

For general considerations the total count of mayonnaise and salad dressings should be low. There should be no coliform, thermophilic, or lipolytic bacteria present. There should be less than 5 yeasts per gm. The type of yeast is important. Yeasts belonging to the genus *Zygosaccharomyces* have been found to cause spoilage in these products. Molds should not exceed 5 per gm.

The presence of excessive numbers of any of the above spoilage organisms indicates improperly cleaned and sanitized machinery and/or the use of contaminated ingredients in making these products.

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CHAPTER XIII

MEAT AND MEAT PRODUCTS

Meat appears on the market in many forms and microbiological methods are needed to assay for quality, detect spoilage, and determine the source of any quality defects. As a result of various methods of producing, processing, and handling meat products, some meat foods act as selective or enrichment media for specific types of microorganisms. Therefore, it seems logical to classify these products into groups that present similar microbiological problems. Attention is called to the lower temperature of incubation (21° F) used in this chapter. Since most meat products are held under refrigeration the bacterial flora are composed largely of cold tolerant types of organisms, and it is believed that a more realistic interpretation of results can be made by use of the lower temperature of incubation for those products requiring refrigeration.

CLASSIFICATION

I. Fresh Meats

These products do not contain curing agents or preservatives and receive no heat treatment. Preservation is entirely dependent upon good sanitation and adequate refrigeration. As a result, for meats that are not intended for canning, the principal microbial flora of importance are restricted generally to organisms that are able to grow at refrigeration temperatures.

Fresh meats may be further subdivided into carcasses, cuts, and ground meat, including hamburger or fresh pork sausage. Carcasses, if properly handled, should have a low interior bacterial count and virtually all of the microorganism should be confined to the surface. Cuts of meat are subjected to additional handling and consequent contamination, but the microorganisms are also confined primarily to the surface. Ground meat is subjected to even more handling and grinding and the contaminants distributed throughout the product.

II. Cured Meats and Sausage Products

By the addition of sodium chloride, nitrate, nitrite, and sugar, meats are cured to prepare a variety of foods, such as hams, bacon, etc. Some may be comminuted in their manufacture, such as frankfurters, bologna, and a number of other sausages, including table-ready meats. Most such products are also subjected to a smoking and/or cooking procedure that kills the vegetative microorganisms in and on the product. The curing agents, primarily the salt, tend to restrict the potential spoilage flora to Gram-positive microorganisms that are relatively salt tolerant. These include various lactic acid bacteria (lactobacilli, leuconostoc, microbacteria, and streptococci), micrococci, members of the genus *Bacillus*, and yeasts. If the product has been adequately processed, these microorganisms, with the exception of the spore formers, are restricted to the surface and represent contaminants that were acquired after the heat processing.

III. Canned Meats

These may be prepared from either fresh or cured meats and may be either semipreserved or fully processed. These latter types of products are stored without refrigeration and any spoilage is due to either spores that survived processing or microorganisms that entered a leaky can. The semipreserved products, such as canned hams, are perishable and must be stored under refrigeration. Spoilage may be due to vegetative cells that survived an inadequate processing or to spores that germinated due to inadequate refrigeration of the finished product. An inadequately processed canned ham or luncheon meat may contain large numbers of lactic acid bacteria without any apparent swelling of the can. When inadequately refrigerated the cans may swell due to growth of aerobic spore formers.

IV. Vinegar Pickled Meats

These products, such as pickled pigs' feet and pickled sausages, generally depend on acidity and an airtight package to prevent spoilage. The most common spoilage is turbidity of the pickle due to extensive growth of lactic acid bacteria.

V. Fermented Sausages

Such meat foods as thuringer, summer sausage, cervelat, Genoa salami, etc., depend upon a lactic type of bacterial fermentation and a

relatively low moisture content for preservation. Generally, they are not subjected to a severe heat processing and thus should be expected to contain large numbers of viable lactic acid bacteria. Their manufacture may also be dependent upon other bacteria to reduce nitrate to nitrite as part of the curing process. However, since there are many varieties of such sausages and many methods of production, it is usually necessary to learn these details concerning the specific product in question in order to interpret the laboratory findings.

GENERAL METHODS

I. Sampling

Care should be exercised in taking samples to make them representative of the items to be tested and to make certain that no contamination or change occurs prior to examination. Take samples under aseptic conditions using sterilized knives, scalpels, forceps, etc. A brace and sterile bit may be employed for obtaining samples from frozen meats. Sterilize the cleaned implements used for obtaining samples by alcohol flaming 3 times before each use. Place samples in sterile containers and transport to the laboratory with sufficient speed and refrigeration to prevent microbial multiplication while in transit. Keep frozen samples solidly frozen and transport unfrozen samples under wet ice refrigeration.

Surface samples may be taken by scraping with a spatula, or broad-bladed knife, or 11 mg or more taken from various positions on the surface using a sterile scalpel and forceps. Relatively thin portions (not over 2 mm in thickness) should be shaved from the surface when using the latter method.

Interior samples may be taken from sausages by scoring the surface with a knife, breaking the sausage by hand, and removing core samples with a sterile knife or scalpel.

Interior samples from solid pieces of meat may be taken by breaking them open where possible, or by sterilizing the surface by searing. or by painting with iodine, or other strong germicidal agent, and then cutting into the center area with a sterile knife and removing samples with a sterile knife or scalpel.

If a particular spot or area is obviously spoiled or discolored, a sample of spoiled material should be taken and, for comparison, also

an apparently unspoiled portion from a similar location on the same product.

If the units can be conveniently handled it is preferable to submit the entire product to the laboratory and do the sampling in the laboratory where it can be examined without delay.

II. Preparation of Samples

Emulsify 11 gm of sample in 99 ml of a sterile diluent in a sterile, mechanical blender cup. The samples should be blended for not more than 3 min in order to avoid overheating of the sample.

Sterile distilled water is satisfactory as the first diluent, but a phosphate buffered dilution blank (23D) is recommended for the subsequent dilutions.

If many samples are to be tested routinely and insufficient blender cups and bases are available for emulsification of the sample, it will be necessary to pass the sample through a sterilized and chilled food grinder followed by vigorous shaking of weighed portions of ground meat in dilution bottles containing 25 gm of glass chips. The chips should be of a size which will pass through a ½-in. mesh wire cloth, but retained by a ¼-in. mesh wire cloth.

Shaking can best be accomplished by using a mechanical paint mixing machine, or other mechanical shaker, but shaking by hand can be used. In either instance a 3-min period of agitation is recommended.

MICROBIOLOGICAL EXAMINATION

I. Fresh Meats

A. *Total Count*

In most instances it is desirable to test both surface and interior meat samples.

1. Transfer appropriate dilutions to Petri plates, in duplicate, and pour with total plate count agar (64).

2. Incubate at 21° C for 72 hr. To obtain more prompt results incubation may be carried out at 32° C for 2 days, but in so doing it should

be realized that some meat samples examined may contain microorganisms which fail to grow at this higher temperature.

3. Count colonies and report as total bacteria per gm of product.

B. *Yeasts and Molds*

Only surface samples should be tested for yeasts and molds.

1. Transfer appropriate dilutions to Petri plates, in duplicate, and pour with potato dextrose agar, acidified (51).

2. Incubate at 21° C for 5 days. Observe at 3 days in order to count if plates are likely to be overgrown. It is realized that all yeasts found on meat will not develop on the recommended medium, but at this time no other selective medium has been sufficiently tested to warrant recommendation.

II. Cured Meats and Sausage Products

A. *Total Count*

Both surface and interior samples should be tested.

1. Transfer appropriate dilutions to Petri plates, in duplicate, and pour with total plate count agar (64) or APT agar (5). The latter is recommended where lactic acid bacteria are suspected as causing spoilage.

2. Incubate at 21° C for 3 days. If speed is mandatory plates may be incubated at 32° C and observed at 1 and 2 days.

3. Count colonies and report as total numbers per gm of product.

B. *Yeasts and Molds*

Surface samples only should be tested for yeasts and molds. Proceed as under I, B, above.

C. *Greening Bacteria*

1. Transfer appropriate dilutions to Petri plates, in duplicate, and pour with APT agar (5), or use such plates if prepared under II, A, 1.

2. Incubate at 21° C for 3 days. Pick colonies suspected of being lactic acid bacteria and inoculate into APT broth (5).

3. Incubate for 24 hr at 32° C and streak a loopful of broth on the

aseptically-cut surface of a frankfurter, or other cured meat product, held in a sterile Petri plate containing a sterile filter paper moistened with 2 ml of sterile distilled water to prevent drying.

4. Incubate inoculated meat at 32° C for 18–24 hr and observe for greening of the meat surface. Uninoculated sausage slices should be similarly incubated for control purposes.

III. Canned Meats

For canned meats that are intended to be commercially sterile, examine according to the procedures recommended in Chapter IV entitled "Canned Foods." For semiperishable canned hams or spiced luncheon meats examine the contents of aseptically opened cans as follows:

A. *Total Count*

Test both surface and interior samples. For canned hams obtain interior samples from vicinity of the boned out area.

1. Transfer appropriate dilutions to Petri plates, in duplicate, and pour with total plate count agar (64) or APT agar (5).

2. Incubate at 32° C for 3 days.

3. Count colonies and report as total numbers per gm of product.

B. *Gas Formers*

1. Pick colonies from total plate count agar (64) or APT agar (5) plates and inoculate, in duplicate, into Jensen's medium (28) according to the method described by Jensen.[4] Pay particular attention to colonies having the appearance of *Bacillus*.

2. Incubate one set of duplicates at 37° C for 3 days for mesophilic types and the other set at 55° C for 2 days for thermophilic types.

3. Observe for trapped gas bubbles in the medium and report as positive those tubes showing gas.

IV. Vinegar Pickled Meats

A. *Total Count*

1. If spoilage is obvious, prepare dilutions from liquor from the sample and transfer to Petri plates, in duplicate. If the sample appears normal, transfer 1 ml and 0.1 ml aliquots of sample to Petri plates.

2. Pour with total plate count agar (64) or with APT agar (5) if lactic acid bacteria are suspected.

3. Incubate at 32° C for 3 days and report as numbers of bacteria per ml of liquid.

V. Fermented Sausages

A. *Total Count*

Test surface and interior samples.

1. Transfer appropriate dilutions to Petri plates, in duplicate, and pour with APT agar (5) or total plate count agar (64).

2. Incubate at 32° C for 3 days. Count colonies and report as bacterial numbers per gm of product.

B. *Greening Bacteria*

Proceed as under II, C, above.

C. *Nonbacterial Greening*

Greening in fermented sausages due to "nitrite burn" may result from excess bacterial nitrate reduction and will not be detected by the above method. A qualitative test for nitrite may be performed by streaking the product (using pipettes) with sulfanilic acid reagent (C2) and then with alpha-naphthylamine reagent (C2). In cases of "nitrite burn" an intense red color develops on the discolored area. These results may be verified by quantitative analyses.[2] This test does not apply to frankfurters, bologna, or similar unfermented sausages which normally contain residual nitrite in qualitatively detectable amounts.

DIRECT MICROSCOPIC EXAMINATION

The use of a direct microscopic examination will give immediate information concerning the relative microbial population and the predominant types of microorganisms in the samples. If the results of plating do not correlate with the microscopic observations, an attempt should be made to determine whether the unrecovered microorganisms (1) were killed during the processing of the product, (2) died during subsequent storage, (3) were killed during the mixing and dilution of the sample, or (4) were unable to initiate growth in the plating medium under the conditions of incubation employed. It must be remembered

that a direct microscopic examination is generally useful only for samples having a microbial population well above 1,000,000 per gm.

1. Prepare a smear on a microscope slide by transferring 0.01 ml of the 1:10 dilution to the slide with a Breed pipette [1] and spreading over 1-sq cm area.

2. Make a Gram stain (B3). If the sample has a high fat content, rinse the fixed smear with xylol before staining.

3. Observe under the microscope and count 100 fields. If the count is being made for only screening purposes, a lesser number of fields may be counted.

4. Report as number of observed organisms per gm of product.

If a microscopic count is not desired, and only observations for bacterial types and relative populations are in order, material may be scraped from the surface of the sample, emulsified in distilled water, the smear being made from the emulsion and stained as directed above.

pH, SALT, AND NITRITE DETERMINATION

Determine the pH of the sample from an aliquot of the 1:10 dilution using a glass electrode pH meter. In case of spoilage the results will give some ideas as to the type of the predominant microorganisms. For example, extensive growth of lactic acid bacteria will lower markedly the pH of products containing sugar; whereas extensive growth of pseudomonads and some micrococci may raise the pH.

Salt and nitrite analyses are useful in determining the cause of certain types of spoilage of cured meat products. For example, the combination of high nitrite and low pH resulting from microbial action can cause a greening of cured meats known as "nitrite burn." Without a nitrite determination it may be difficult to distinguish this type from greening produced by peroxide that is formed oxidatively by some lactic acid bacteria. Analyses for salt and nitrite are to be performed according to the methods described by the Association of Official Agricultural Chemists.[2]

INTERPRETATION OF RESULTS

In many instances correct interpretation of results may be even more critical than the quantitative accuracy of the laboratory procedure.

Obviously, the present chapter cannot provide sufficient background information for interpreting results in many specific problems, but can only provide a few basic principles. For a broad background of information the reader is referred to the book, "Microbiology of Meats," by Jensen.[4]

In addition, one should learn as much as possible concerning the methods of producing and processing the product to be examined and strive to obtain a reasonable history of the specific sample from the time it was processed.

Slime on meat products may be the result of excessive growth of almost any microorganism. The slime normally consists of confluent microbial growth, although an extreme amount of gummy slime may be polysaccharide produced from sugar by certain lactic acid bacteria.

Pseudomonads and related microorganisms grow fairly rapidly on fresh meats even under adequate refrigeration. Therefore, high counts of pseudomonads and related bacteria are not necessarily to be interpreted as evidence of poor sanitation, although these bacteria may cause sliming or offensive odors upon extensive holding. Adequate refrigeration is the only permissible method that will retard the growth of these microorganisms that may contaminate the meat surface.

In cases of discoloration of meat pigments be alert for nonbacterial as well as bacterial causes. The cured meat pigment is very sensitive to oxidation and may be discolored by oxygen (light catalyzed), hypochlorite, excess nitrite, rancid fat (as in frozen cured meat products), or by metal-catalyzed auto-oxidation.

A dry white deposit on the surface of cured meats is often called mold, but it is more often due to yeasts or micrococci, or may be nonmicrobial. The latter type may include phosphate crystals, salts from hard water, lactose crystals, or similar compounds.

The microorganisms on the surface of cured products that are subjected to a heat process generally represent contamination of the product after processing and bacterial growth during subsequent holding. Microorganisms in the interior of such products are generally those that survived the heat processing and may have multiplied during the holding period.

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CHAPTER XIV

POULTRY MEAT

The widespread sale and use of poultry in many forms and the need for good sanitation in the dressing and holding of these products make the establishment of uniform microbiological methods desirable. Products of high sanitary quality are cleaner, more healthful, of better appearance, and better keeping quality.[1] The methods presented here should be useful in quality control in dressing plants and the estimation of sanitary quality of items found in the market.

Poultry may be marketed as dressed, eviscerated, or cut-up types of chickens, turkeys, or other fowl. These may be in the form of fresh, chilled, of frozen products. A large amount of cooked poultry meat is marketed as canned products or as ingredients in several types of frozen products.

With few exceptions bacterial contamination and growth is a surface phenomenon in raw poultry products. It results from the resident flora of the skin of the birds, the handling during dressing and processing and growth during the holding period. Bacterial examination of the surface of the birds during processing reveals the efficiency of sanitary practices employed and during the holding or storage period indicates the general conditions under which they were held.

The methods presented here are designed to obtain results without sacrifice of the product being examined, and so that if necessary a considerable part of the work can be done in the processing plant, in the warehouse or store, and the final work carried out in the laboratory.[3]

SAMPLING

I. Dressed and Eviscerated Poultry

Select dressed or eviscerated birds at random from the processing line, or from containers, to be representative of the lot. Place in sterilized containers or wrap in sterilized paper or cloths. Polyethylene

or pliofilm bags of convenient size may be used to good advantage. As received such bags are sterile or have very low bacterial counts. Contamination can be determined by making control counts on the package of bags as received.

Handle and transport samples in a sanitary manner, keeping unfrozen samples refrigerated at all times and frozen samples solidly frozen during transportation to the laboratory and until ready for examination.

Do not allow the surface of the poultry to dry before the examination.

II. Cut-Up Poultry

Select cut-up poultry parts so as to be representative of the lot. Thigh or breast pieces may be used as representative of the cut-up bird as they are from different parts of the body and there is a relatively large skin area available for testing. Select poultry parts from different portions of the package.

PREPARATION OF SAMPLES AND SAMPLING MATERIAL

I. Dressed and Eviscerated Poultry

Leave all samples in the freezer or refrigerator until ready for the examination, then place in warm air where the sample can thaw or warm quickly. It is not necessary that the entire bird be thawed, but the surface areas should be flexible and free of ice. Arrange the poultry carcass for easy access to a broad skin area of the breast, thigh, or back so sampling may be done with a minimum of effort and handling. Do not allow surface to become dry.

Prepare small cotton swabs on round wooden applicators. Insert swabs, 3–5, with cotton end down into test tubes of suitable size, plug with cotton, and sterilize.

Place several circular templates in Petri plates and sterilize. A convenient template is a waxed paper gasket commonly used in a 2-in sanitary milk or egg pipe line.[1] The open center of this gasket has an area of 12 sq cm. Due to the convenient size, shape, and flexibility this template is very useful in sampling the surface areas of poultry and may be used and resterilized repeatedly.

Prepare and sterilize 99-ml saline dilution blanks (23E), using bottles with rather wide openings so that swabs may be inserted easily.

As an alternate, place aluminum Gunderson "spot plates" [2] in Petri plates and sterilize. Fill the spot plates to the brim with sterile total plate count agar (64), using approximately 12 ml, by means of a large bore pipette. The spot plate presents a 16-sq cm agar surface. When solidified the agar should present a surface slightly above the rim of the spot plate so it may be pressed firmly against the skin of the bird.

II. Cut-Up Poultry

Arrange selected poultry parts for ready access for culturing. Do not allow surface to become dry.

Prepare and sterilize 99-ml saline dilution blanks (23E) using ordinary dilution bottles.

Prepare and sterilize saline dilution water in 1,000-ml portions or other predetermined amounts for making shake cultures. Obtain polyethylene or pliofilm bags of approximately 8 in. by 12 in. for making shake cultures. Open the package and store under aseptic conditions. As a rule these bags are sterile or contain spore-forming bacteria in such small numbers as to add a negligible amount to the total count. To check for contamination select 3 bags from the package and run control counts of the interior surface by pouring the contents of a 99-ml saline dilution blank into the bag, shaking and pipetting 10-ml into each of 3 Petri plates. Pour with total plate count agar (64) and incubate at 32° C for 72 hr.

Provide sterile tongs for handling the pieces of poultry.

MICROBIOLOGICAL EXAMINATION

I. Dressed and Eviscerated Poultry

Select a sterile template with sterile forceps, place firmly against the skin surface of the breast of the bird, and hold with fingers placed on the outside edge of the template.

Remove a sterile swab from a test tube and thoroughly swab the entire skin surface exposed in the opening in the template, rolling the swab

during the operation to expose all surfaces. Remove the cap or cotton plug from a dilution bottle and insert the swab about 2 in. within the neck of the bottle. Break the tip off by bending the applicator sharply against the glass dropping the cotton tip into the dilution water. If the skin surface is dry, moisten the swab in the dilution blank prior to swabbing the surface.

Repeat the process by swabbing the skin surface of the thigh or other selected area.

A. *Total Count*

Shake the dilution blank containing the swab on a shaking machine or violently give 50 shakes by hand. Prepare serial dilutions from this blank.

Pipette 1 ml of appropriate dilutions into Petri plates, in duplicate, and pour with total plate count agar (64).

Incubate 72 hr at 32° C and count the colonies that develop. Report the average as the total count per sq cm of skin surface, indicating the area sampled.

Alternate Method:

Remove a Gunderson spot plate [2] from its Petri plate holder, place the agar surface against the skin surface, and press gently but firmly in order to make good contact. Remove from the surface by lifting at one edge so as not to smudge the surface or to pull the agar from the plate.

If the surface count is low the spot plate may be returned to the Petri plate and incubated for 48 hr at 32° C. Count the colonies on the 16-sq cm area with a colony counter lens and report as number of colonies per sq cm of skin surface. Colonies tend to grow together upon longer incubation and counting becomes difficult or impossible.

In many cases the count is too high to be determined by this method and Gunderson, et al.,[3] have devised a variation that overcomes this difficulty. The spot plate may be applied in the processing plant or in the laboratory and the following technic applied:

Aseptically remove the agar disk from the spot plate and transfer to a sterile blender jar containing 150 ml of sterile saline solution. Thus,

each ml of dilution represents 0.1 sq cm of agar surface pressed against the skin surface.

Blend for 2 min and allow the material to stand momentarily to disperse the foam. Make serial dilutions and pour plates in duplicate with total plate count agar (64).

Incubate for 72 hr at 32° C. Report the average number of colonies as total count per sq cm of skin surface.

II. Cut-Up Poultry

Cut-up poultry does not present a continuous skin surface and may often present raw flesh and bone over most of the surface, while that portion representing the body cavity is very irregular and difficult of access. Thus the swab technic or the spot plate method do not readily lend themselves to bacterial estimation of such pieces. The method recommended employs a washing technic to overcome these difficulties.[1]

Remove a plastic bag aseptically from the package or other container and open to receive the sample. With sterilized tongs transfer a thigh, breast, or other part to the plastic bag. Add sterile saline water in amounts to the nearest 100 ml approximating 3 times the weight of the pieces of chicken. If a count on a number of pieces is desired all of the pieces may be placed in the plastic bag at once.

Squeeze about ½ of the air from the bag, gather the open end, and twist firmly leaving considerable slack in the bag. Grasp the twisted end of the bag in one hand and the bottom in the other and shake vigorously 50 times.

A. *Total Count*

Prepare serial dilutions by transfer of liquid from the bag to dilution blanks. Transfer 1-ml portions of appropriate dilutions to Petri plates, in duplicate, and pour with total plate count agar (64).

Incubate for 72 hr at 32° C and count the colonies. Using the weight of the piece refer to Figure 1 for the approximate surface area. Multiply count by the dilution factor and report average as total count per sq cm of surface of piece of poultry, indicating the particular type of piece used.

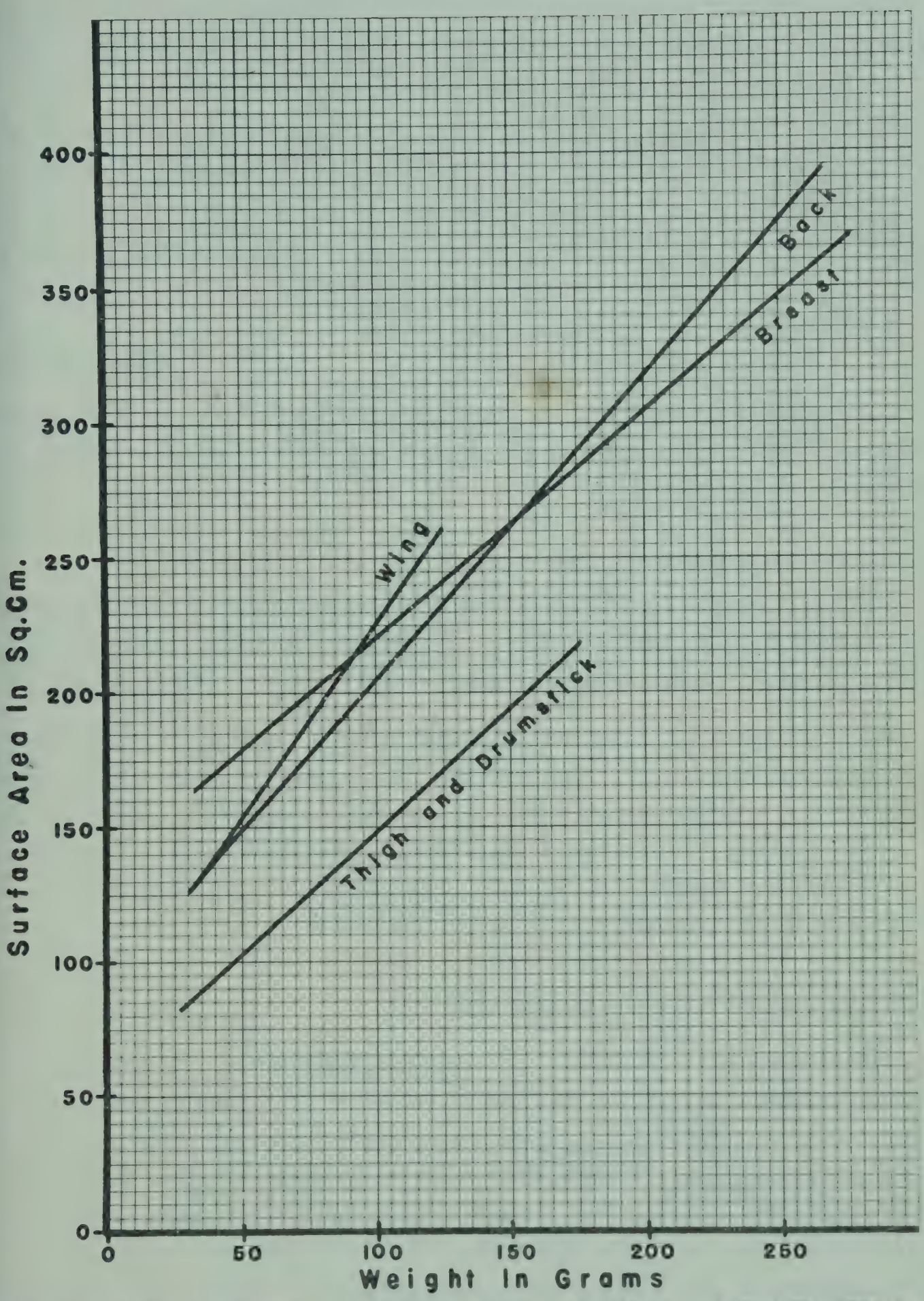

Figure 1—Approximation of Surface Areas of Pieces of Cut-Up Chicken

APPROXIMATE SURFACE AREAS OF CHICKEN PARTS[4]

Since chicken pieces are very irregular in shape and are 3 dimensional, it is difficult for the laboratory worker to relate his findings to a realistic attribute of the piece. When using the shake method of making bacterial counts the reporting of the bacterial count per piece, or for a given weight of piece, does not relate the count to the surface area from which the bacteria were washed. The bacteria are on the surface and not in the meat and, therefore, it is most logical to report as bacterial numbers per sq cm of surface area.

In order to assist the laboratory worker in determining the surface area of various pieces of chicken a series of curves are presented for use. Figure 1 presents a means of approximating or estimating the surface areas of like pieces of chicken according to their respective weights.

A. *Approximation of Surface Area*

After the sample of dilution water for plating has been removed from the plastic bag, remove the piece of chicken, blot with a sterilized or clean towel to remove excess moisture, and weigh to the nearest gm.

From the point on Figure 1, corresponding to the weight of the piece, draw a line upward to intersect with the curve representing the type of piece of chicken under test. From this point draw a line to the "Surface Area" ordinant and read the approximate surface area in sq cm. Using this figure, and the total count obtained, calculate and report numbers of microorganisms per sq cm of surface.

INTERPRETATION OF RESULTS

Abnormally high bacterial counts are an indication of poor handling practices, lack of proper sanitation, or lack of temperature control. Counts from freshly killed birds are much lower than from chilled stored birds or from thawed, frozen birds.[3] If the count is high, from a few hundred thousand to several million, it is an indication that the birds have been improperly handled, held at temperatures above that of melting ice, or at fluctuating temperatures. Clean birds or parts packed in ice, or held at 32°–34° F, should have a shelf life of approximately 4 days without a marked rise in surface count. Birds processed under in-plant chlorination[1] should have low counts and have a longer shelf life.

If the temperature is allowed to rise even a few degrees, the count will rise rapidly. If birds are not properly chilled after dressing and are held at room temperature for only a few hours before being placed under chill temperature, the count will rise rapidly.

If the surface of the skin or exposed flesh is slick or slimy to the touch, it is likely that the count will be in the millions per sq cm of surface and is an indication of long holding in the unfrozen state or at temperatures above that of melting ice. Slick poultry generally has an odor or it will develop quickly after the slick stage.

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CHAPTER XV

SPICES

The bacteriological examination of spices is important, since they are used as ingredients in many types of prepared foods. It is known that most types of raw spices are high in bacterial count [1] and may thus contribute to the spoilage of food products in which they are used, as for example, in meat products which are given a low process, such as sausages, luncheon meats, etc.[2] Spices which have undergone a sterilization treatment may have a low microbial count compared to untreated spices. Spice oils are usually sterile.

Spices may be divided into "whole spices," such as all-spice, cloves, cinnamon sticks, etc.; "ground spices," such as pepper, coriander, ground cloves, etc.; and "bulky spices," such as bay leaves, herbs, etc.

SAMPLING AND PREPARATION

In sterile jars collect at least 50 gm from each of 3 containers, seal tightly, and send to the laboratory with the essential data identifying the sample. If spices are in small packages send unopened containers to the laboratory.

Weigh the spices on sterile paper or directly into a sterile dilution bottle using a previously flamed spatula. The amount of spice examined will depend upon the bacterial count and the character of the spice. The following amounts are suggested:

Whole spices	10 gm
Bulky spices	2 "
Ground spices	1 "

Add sterile water to the 100-ml mark of the dilution bottle and shake for 5 min. Prepare serial dilution in the usual manner using 99-ml dilution blanks (23A). Each dilution should be agitated for 1 min and coarse particles allowed to settle out before transferring to the next dilution bottle or to plates.

MICROBIOLOGICAL EXAMINATION

I. Total Count

Transfer suitable aliquots in duplicate to Petri plates and pour with total plate count agar (64). Incubate for 48 hr at 32 ° C. Report as total count per gm of spice.

II. Yeasts, Molds, and Acid-Tolerant Bacteria

Transfer suitable aliquots in duplicate to Petri plates and pour with potato dextrose agar, acidified (51) or malt agar (36). Incubate at 32° C for 5 days and report as number of colonies per gm. Observe plates at 3 days in order to make counts if molds are spreading over the plates. Tomato juice agar (63) may be used to advantage for acid-tolerant bacteria.

III. Aerobic Thermophiles

Transfer suitable aliquots in duplicate to Petri plates and pour with dextrose tryptone agar (20). Incubate for 48 hr at 55° C and report as thermophilic bacteria per gm of spice.

IV. Aerobic Spore Formers

A. *Mesophilic Types*

After making dilutions for the other types of counts, boil the initial spice suspension for 5 min and restore evaporation loss with sterile water. Plate appropriate dilutions using dextrose trytone agar (20). Bromcresol purple indicator (C5) may be incorporated in the medium if the counting of acid-forming types is desired. After the medium has solidified stratify with a thin layer of plain agar (49) to reduce interference from spreaders. This step is especially recommended for "sterilized" spices because of surviving spores. Incubate at 32° C for 72 hr and report as number of spores per gm.

For the detection of aerobic spore-forming, gas-producing types inoculate 5 tubes of Jensen's medium [2] (28) with serial dilutions and incubate at 37° C for 72 hr. Refer to Table 1, Chapter XVI, and report as most probable number of organisms per gm of product. Incubation at 49° C (120° F) may be employed for more rapid gas formation.

B. *Thermophilic Flat-Sour Types*

Advantage is taken of the greater heat resistance of the flat-sour spores to eliminate lower heat-resistant mesophilic types. Transfer 10 ml of spice suspension to 100 ml of melted dextrose tryptone bromcresol purple agar (21), mix and autoclave for 10 min at 5-lb steam pressure. Raise the pressure rapidly and decrease it as rapidly as possible in order not to expose the medium to high heat any longer than necessary. Cool to about 45° C, distribute agar among 5 plates, and allow to solidify. Incubate at 55° C for 48 hr and count the typical colonies. Calculate and report as spores per gm of sample.

V. Anaerobic Spore Formers

A. *Total Count*

From the above serial dilutions transfer 1-ml aliquots to Petri plates and pour with total plate count agar (64). Incubate under anaerobic conditions for 3 days at 32° C. Report as total number of anaerobic spores.

Oval test tubes, modified by a round neck,[3] may be used instead of Petri plates. Use tubes of recently melted and rapidly tempered total plate count agar (64) and after chilling to solidify the agar, fill the neck of the tube with reduced methlyene blue agar (44). Incubate 48–72 hr at 32° C and count the colonies using a plate counting lens.

B. *Mesophilic-Putrefactive Types*

Transfer 1 ml of appropriate dilutions of boiled spice suspension to each of 5 freshly steamed tubes of liver broth medium[4] (32) and stratify with nutrient agar (45), solidify, and incubate at 32° C for 5 days. Record number of positive tubes and refer to Table 1, Chapter XVI. Report as most probable number of putrefactive spores.

C. *Thermophilic Types—Not Producing Hydrogen Sulfide*

Transfer 1 ml of appropriate dilutions of boiled spice suspension to each of 5 freshly steamed tubes of liver broth medium[4] (32) and stratify with nutrient agar (45). Solidify, preheat to 55° C, and incubate at 55° C for 48 hr. Record number of tubes showing growth and refer to Table 1, Chapter XVI. Report as most probable number of spores.

D. *Thermophilic Types—Producing Hydrogen Sulfide*

Transfer 1 ml of appropriate dilutions of boiled spice suspension to each of 5 oval tubes [3] of sulfite agar (59), freshly melted and cooled to about 45° C. Mix thoroughly, solidify, preheat to 55° C, and incubate for 48 hr at 55° C. Count the typical black colonies and report the average as number per gm of spice.

INTERPRETATION OF RESULTS

Spices are generally used as flavoring ingredients in food products and if the bacterial populations of specific organisms are excessive may contaminate the product to a point where spoilage may occur. For example, the population of thermophilic flat-sour spores and thermophilic types not producing hydrogen sulfide should be low if the spices are to be used in canned products. Spices to be used in canned cured meat products should be free of organisms producing gas in Jensen's medium. Such types of organisms do not have the same importance in noncanned products.

Spices showing very high counts are likely to be of low sanitary quality and indicative of conditions of production and handling. Bacterial counts may be used as a guide in designating the point of use for certain lots of spices.

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CHAPTER XVI

SANITATION INDEXES

The cleanliness of food is considered essential by the consumer. He places a trust in the food producer, processor, and handler that the food presented to him has been produced under sanitary conditions and is as free of contamination as possible. There has been considerable controversy over the types of organisms that are most indicative or can best be used in describing the sanitary quality of a food product. This is also true of the significance of numbers of such organisms. Of the suggested indexes of sanitary quality of foods, three have been given the most consideration: *Escherichia coli*, coliforms, and enterococci. The methods for the detection and enumeration of these types of organisms are set forth in this chapter.

The laboratory detection of microorganisms of these groups is only an indication of a situation which existed at some point along the path that the food has traveled. It is only when adequate history of the product is known that the laboratory information may be used to pin point the source and cause of the trouble. Recontamination of processed products through handling, or the holding of food products at temperatures ideal for bacterial multiplication, are examples of conditions that must be sought out and corrected. The presence of large numbers of these types of organisms is not necessarily indicative of an immediate health hazard, but it does indicate lack of good sanitary practices and it raises a warning flag that the conditions which brought about the contamination could easily give rise to spoilage, loss of quality, or create a health hazard. However, it must be remembered that certain members of the coliform group are to be found in certain fermented types of food and may even play a part in the process. Examples are dried egg albumen,[1] cucumber pickle stock,[2] certain cheeses,[3, 4] sauerkraut,[5] and cured olives.[6–8] Their presence in many fresh fruits and vegetables usually cannot be interpreted as indicative of insanitation.[9]

I. PREPARATION OF SAMPLE

The sample should be prepared for examination by methods set forth in the various chapters, making sure that a representative, homogeneous preparation is obtained for inoculation. It has been pointed out by Larkin, Litsky, and Fuller [10] that under certain conditions the use of a blender is desirable in making examinations for coliforms and enterococci. They show that more accurate results can be obtained and detections made more frequently by the use of the blender than shaking by hand. The blender should not be run for longer than 2 min. Jones and Ferguson,[11] however, have shown a decrease in counts due to the use of the blender in certain products.

Highly acid products, such as citrus juices and concentrates, tomato juice and acid pickles, should be neutralized with sterile sodium hydroxide to a pH range of 5.5–6.0 before testing for coliforms.

II. DETECTION AND ENUMERATION OF THE COLIFORM GROUP [12]

1. Detection by Liquid Media

Under certain conditions it is desirable to determine merely the presence or absence of such organisms, or the relative amount of contamination, as in routine sanitary control of food plant operations.

A. *Presumptive Test*

Routine plant control or screening for sanitary quality of foods:

1. Inoculate 1 ml of appropriate dilutions into a fermentation tube of brilliant green lactose bile broth (13) or lauryl tryptose broth (30).

2. Incubate at 35° C and examine after 24 and 48 hr. The presence of gas in the tube indicates a positive presumptive test for members of the coliform group.

B. *Completed Test*

For a completed test proceed as follows:

1. Select the last 2 dilution tubes showing a positive presumptive reaction. After shaking gently, streak the "dry" surface of Endo (24) or EMB(31) agar plates with material from the tubes using a curved needle and being careful to avoid picking up scum from the broth.

2. Incubate the plates for 24 hr at 35° C and observe for typical coliform colonies.

3. Fish typical colonies into fermentation tubes of brilliant green lactose bile broth (13) or lauryl tryptose broth (30). At the same time streak material from the colony on a nutrient agar slant (45).

4. Incubate for 24 hr at 35° C. Observe for presence of gas as evidence of coliforms.

5. If the fermentation tube shows gas, make a smear on a microscope slide from the agar slant. Make a Gram stain (B3) and observe under the microscope. The presence of Gram-negative, nonspore-forming short rods on the slide and a positive reaction in the broth tube constitutes a positive completed test for coliforms.

2. Enumeration of Coliform Group

A. *Solid Media*

1. Transfer 1-ml portions of appropriate decimal dilutions to duplicate Petri plates and pour with violet red bile agar (73) or desoxycholate lactose agar (16). Mix the medium and the inoculum thoroughly and allow agar to solidify. Distribute an additional 4–5 ml of medium over the surface of the agar to inhibit surface and spreader-type growth.

2. As soon as agar is solidified invert plates and incubate for 18–24 hr at 35° C. Count the dark red colonies, at least 0.5 mm in diameter, and report the average as numbers of coliform colonies.

3. If there is need to confirm the colonies as true coliforms, transfer material from typical colonies to fermentation tubes of brilliant green lactose bile broth (13) or lauryl tryptose broth (30). If the sample contained fermentable carbohydrates confirmation is necessary.

4. Incubate for 24 hr at 35° C and observe for gas. If the tube is positive make a Gram stain (B3) and examine under the microscope. A positive gas production and the presence of Gram-negative, nonspore-forming short rods confirm the judgment on the colonies under 2.

B. *Liquid Media*

1. To enumerate coliform organisms using liquid media inoculate each decimal dilution into 5 fermentation tubes using 1-ml portions. Use brilliant green lactose bile broth (13) or lauryl tryptose broth (30).

2. Incubate at 35° C and examine after 24 and 48 hr. Observe for gas and select the last dilution in which all 5 tubes are positive and the next 2 dilutions. If this is not possible, select the last 3 positive dilutions. Record the number of positive tubes in each dilution and refer to Table 1 in this chapter. Report the result as estimated or most probable number of coliforms present. Thus, if the number of tubes in the last 3 positive dilutions were 5–3–2, from the table, the MPN would be 140. This figure divided by 100 and multiplied by the dilution of the middle set of tubes is equal to the most probable number of coliforms per gm of sample. In cases where "skips" are encountered or where the end point is not clear-cut the worker must resort to "best judgment" in selection of dilutions to use. For further information refer to page 384, "Standard Methods for the Examination of Water, Sewage, and Industrial Wastes." [13] It will be noted that the temperature of incubation is given as 35° C to conform to that required in the book of "Standard Methods."

3. To confirm the presence of coliforms streak material from each tube in the highest 3 dilutions which show gas on the surface of Endo agar (24) or EMB agar (31) using a curved needle and taking care to avoid picking up scum from the tube.

4. Incubate the plates for 18–24 hr at 35° C and observe for typical coliform colonies.

5. Further confirmation may be made by means of a Gram stain (B3) made directly from the colony.

III. DETECTION AND ENUMERATION OF ESCHERICHIA COLI

Escherichia coli has long been used as an index of fecal contamination of food, milk, and water. The detection and enumeration of this specific type of organism may be used to determine the sanitary history of certain foods, to trace the recontamination of food during processing through handling by man, or perhaps the detection of food contamination by animals during storage.

Where it is desired to differentiate this organism from other members of the coliform group proceed as follows:

1. Detecton of *E. coli* by Liquid Media

A. *Coliform Fermentation Tubes*

1. *E. coli* may be differentiated as a specific organism during the tests for the coliform group by selecting the positive fermentation tubes used for coliforms under II, 1, A, and streaking liquid on the "dry" surface of EMB agar (31). Use a curved needle and streak so as to obtain well isolated colonies.

2. Incubate for 18–24 hr at 35° C. Observe for slightly raised colonies, 2–3 mm in diameter, having flat to concave tops, a dark center and having a greenish metallic sheen.

3. Report dilutions positive that show typical colonies. If further confirmation is required, determine the IMViC reactions of the colonies.

B. *Boric Acid Lactose Broth*[14]

Levine, et al.,[15] point out that coliforms and *E. coli* can be determined simultaneously by employing a coliform medium as a primary medium incubated at 35° C and followed by a boric acid medium incubated at 43° C. These authors particularly stress this as a secondary confirmatory medium.

1. After readings of the coliform fermentation tubes have been taken under II, 1, A, above, transfer a loopful of broth from each positive tube to a tube of buffered boric acid lactose broth (15).

2. Incubate at 43±0.5° C in a thermostatically controlled bath and examine after 24 and 48 hr. Tubes showing gas may be considered confirmed for *E. coli.*

3. If there is need for further confirmation streak material from the tubes on EMB agar (31), and after incubation for 18–24 hr at 35° C observe for typical *E. coli* colonies.

2. Enumeration of *E. coli*

A. *Coliform Fermentation Tubes*

An approximation of the density of *E. coli* can be made from the fermentation tubes used for obtaining MPN values for coliforms in II, 2, B.

1. Streak material from each positive tube in the last 3 positive dilutions on the "dry" surface of EMB agar (31).

2. Incubate for 18–24 hr at 35° C and observe for slightly raised colonies, 2–3 mm in diameter, having flat or concave tops, dark centers, and a greenish metallic sheen. If further confirmation is required, determine the IMViC reactions of the colonies.

3. Record the number of tubes positive for *E. coli* in each dilution and refer to Table 1 for the MPN. In the example given under II, 2, B, above, the reading was 5–3–2 tubes positive for coliforms. If now the reading is 4–2–1 positive for *E. coli*, we find the MPN to be 26. This figure divided by 100 and multiplied by the dilution of the middle set of tubes is equal to the most probable number of *E. coli* per gm of sample. Thus, if the 3 positive dilutions were 1:100, 1:1,000, and 1:10,000, then the MPN for coliforms would be 1,400 per gm and for *E. coli* 260 per gm.

B. *Buffered Boric Acid Lactose Broth*

The boric acid medium may be used as a secondary medium [15] to rapidly estimate the density of *E. coli* in a sample by the MPN method.

1. From the positive fermentation tubes used under II, 2, B, inoculate fermentation tubes of buffered boric acid lactose broth (15) with a loopful of broth.

2. Incubate at 43±0.5° C using a thermostatically controlled bath and examine after 24 and 48 hr for gas. The presence of gas indicates a positive reaction.

3. Relate to the lactose fermentation tubes and record the number of confirmed tubes in each of the last 3 positive dilutions. Refer to Table 1 for the MPN figure. This number divided by 100 and multiplied by the middle dilution is equal to the most probable number of *E. coli* organisms per gm of sample.

4. If further confirmation of the presence of *E. coli* is necessary, streak material from each positive tube of the boric acid broth on the "dry" surface of EMB agar (31). After incubation for 18–24 hr at 35° C observe for typical *E. coli* colonies. Due to the selective action of the boric acid medium this step is generally not necessary.

C. *Solid Media*

An estimate of the number of *E. coli* on the coliform plates used in II, 2, A can be made by making an "*E. coli* count" [16] of the colonies.

1. EMB Technic

a) After making the count of coliform colonies on violet red bile or desoxycholate lactose agar plates, II, 2, A, pick at random 10 representative coliform colonies from the dilution showing at least 30 such colonies. Streak on the "dry" surface of EMB agar (31) in such a way as to obtain well isolated colonies.

b) Incubate for 18–24 hr at 35° C and observe for typical *E. coli* colonies. Record the number of colonies confirmed for *E. coli* and express as a percentage of the total colonies picked. Multiply the total number of coliform colonies by this percentage to obtain the estimated number of *E. coli* colonies. Thus, if the total coliform count on the plate is 50 and 5 of the 10 colonies picked are confirmed as *E. coli*, then the estimated number of *E. coli* colonies on the plate would be 25. This figure multipled by the dilution is equal to the estimated number of *E. coli* organisms per gm of sample.

2. Boric Acid Lactose Broth Technic

As an alternate method the buffered boric acid lactose broth (15) can be used for confirming *E. coli* colonies on the coliform plates.

a) Pick at random 10 representative coliform colonies from the violet red bile or desoxycholate lactose agar plates from II, 2, A, showing at least 30 such colonies, and transfer to fermentation tubes of buffered boric acid lactose broth (15).

b) Incubate at 43±0.5° C in a thermostatically controlled bath and observe at 24 and 48 hr. Record colonies as *E. coli* confirmed if gas is shown in the tubes.

c) Express the confirmed colonies as a percentage of the total colonies picked and multiply the total coliform colonies by that number. The resultant figure multiplied by the dilution is equal to the estimated number of *E. coli* organisms per gm of sample.

IV. DETECTION AND ENUMERATION OF FECAL STREPTOCOCCI

The presence of fecal streptococci has been suggested as a sanitation index, since they have been shown in various types of products, such as water,[17] milk,[18] frozen foods,[19] eggs,[20] fish and shellfish,[10] etc. Larkin,

Litsky, and Fuller,[10, 19] and Burton [21] indicate that these organisms are more consistently found in frozen food products than the coliforms or *E. coli.* Dack [22] has reported on their significance in foods. It has been shown that these organisms have fairly high heat resistance [23] and that their presence may indicate inadequate heat processing or where adequate heating has been used their presence may indicate recontamination.

When the detection and estimation of the density of the fecal streptococci are desired the following methods are recommended:

1. Detection of Fecal Streptococci

a) Transfer 1-ml portions of selected decimal dilutions of the sample into duplicate tubes of azide dextrose broth (6).

b) Incubate at 35° C and observe for growth after 24 and 48 hr. If growth is evident the tube is considered presumptively positive.

c) Transfer a loopful of broth from each positive tube to a tube of ethyl violet azide broth (26).

d) Incubate at 35° C and observe for turbidity after 24 and 48 hr. If growth is evident, the tube is considered confirmed for fecal streptococci. Report fecal streptococci as present in the last positive dilution or equivalent weight of sample.

e) Further confirmation can be made by making a Gram stain (B3) and observing for Gram-positive streptococci under the microscope.

2. Enumeration of Fecal Streptococci

When an estimate of the density of fecal streptococci is desired:

a) Transfer 1-ml portions from each selected dilution of the sample into 5 tubes of azide dextrose broth (6).

b) Incubate at 35° C. Observe for growth after 24 and 48 hr and consider each tube showing growth as presumptively positive.

c) Transfer a loopful of broth from each positive tube in the last 3 positive dilutions to a tube of ethyl violet azide broth (26).

d) Incubate at 35° C and observe for turbidity after 24 and 48 hr. Record the number of confirmed positive tubes in each dilution and refer to Table 1 for the MPN figure. This number divided by 100 and multiplied by the middle dilution is equal to the most probable number of fecal streptococci per gm of sample.

V. MOST PROBABLE NUMBER

Table of Most Probable Numbers (MPN) per 100 ml of Sample [18]

Using 5 Tubes
With 10, 1 and 0.1 ml Volumes

Pos * 10 1 0.1	MPN	Pos * 10 1 0.1	MPN	Pos * 10 1 0.1	MPN	Pos * 10 1 0.1	MPN	Pos * 10 1 0.1	MPN	Pos * 10 1 0.1	MPN
0 0 0	0	1 0 0	2	2 0 0	4.5	3 0 0	7.8	4 0 0	13	5 0 0	23
0 0 1	1.8	1 0 1	4	2 0 1	6.8	3 0 1	11	4 0 1	17	5 0 1	31
0 0 2	3.6	1 0 2	6	2 0 2	9.1	3 0 2	13	4 0 2	21	5 0 2	43
0 0 3	5.4	1 0 3	8	2 0 3	12	3 0 3	16	4 0 3	25	5 0 3	58
0 0 4	7.2	1 0 4	10	2 0 4	14	3 0 4	20	4 0 4	30	5 0 4	76
0 0 5	9	1 0 5	12	2 0 5	16	3 0 5	23	4 0 5	36	5 0 5	95
0 1 0	1.8	1 1 0	4	2 1 0	6.8	3 1 0	11	4 1 0	17	5 1 0	33
0 1 1	3.6	1 1 1	6.1	2 1 1	9.2	3 1 1	14	4 1 1	21	5 1 1	46
0 1 2	5.5	1 1 2	8.1	2 1 2	12	3 1 2	17	4 1 2	26	5 1 2	64
0 1 3	7.3	1 1 3	10	2 1 3	14	3 1 3	20	4 1 3	31	5 1 3	84
0 1 4	9.1	1 1 4	12	2 1 4	17	3 1 4	23	4 1 4	36	5 1 4	110
0 1 5	11	1 1 5	14	2 1 5	19	3 1 5	27	4 1 5	42	5 1 5	130
0 2 0	3.7	1 2 0	6.1	2 2 0	9.3	3 2 0	14	4 2 0	22	5 2 0	49
0 2 1	5.5	1 2 1	8.2	2 2 1	12	3 2 1	17	4 2 1	26	5 2 1	70
0 2 2	7.4	1 2 2	10	2 2 2	14	3 2 2	20	4 2 2	32	5 2 2	95
0 2 3	9.2	1 2 3	12	2 2 3	17	3 2 3	24	4 2 3	38	5 2 3	120
0 2 4	11	1 2 4	15	2 2 4	19	3 2 4	27	4 2 4	44	5 2 4	150
0 2 5	13	1 2 5	17	2 2 5	22	3 2 5	31	4 2 5	50	5 2 5	180
0 3 0	5.6	1 3 0	8.3	2 3 0	12	3 3 0	17	4 3 0	27	5 3 0	79
0 3 1	7.4	1 3 1	10	2 3 1	14	3 3 1	21	4 3 1	33	5 3 1	110
0 3 2	9.3	1 3 2	13	2 3 2	17	3 3 2	24	4 3 2	39	5 3 2	140
0 3 3	11	1 3 3	15	2 3 3	20	3 3 3	28	4 3 3	45	5 3 3	180
0 3 4	13	1 3 4	17	2 3 4	22	3 3 4	31	4 3 4	52	5 3 4	210
0 3 5	15	1 3 5	19	2 3 5	25	3 3 5	35	4 3 5	59	5 3 5	250
0 4 0	7.5	1 4 0	11	2 4 0	15	3 4 0	21	4 4 0	34	5 4 0	130
0 4 1	9.4	1 4 1	13	2 4 1	17	3 4 1	24	4 4 1	40	5 4 1	170
0 4 2	11	1 4 2	15	2 4 2	20	3 4 2	28	4 4 2	47	5 4 2	220
0 4 3	13	1 4 3	17	2 4 3	23	3 4 3	32	4 4 3	54	5 4 3	280
0 4 4	15	1 4 4	19	2 4 4	25	3 4 4	36	4 4 4	62	5 4 4	350
0 4 5	17	1 4 5	22	2 4 5	28	3 4 5	40	4 4 5	69	5 4 5	430
0 5 0	9.4	1 5 0	13	2 5 0	17	3 5 0	25	4 5 0	41	5 5 0	240
0 5 1	11	1 5 1	15	2 5 1	20	3 5 1	29	4 5 1	48	5 5 1	350
0 5 2	13	1 5 2	17	2 5 2	23	3 5 2	32	4 5 2	56	5 5 2	540
0 5 3	15	1 5 3	19	2 5 3	26	3 5 3	37	4 5 3	64	5 5 3	920
0 5 4	17	1 5 4	22	2 5 4	29	3 5 4	41	4 5 4	72	5 5 4	1600
0 5 5	19	1 5 5	24	2 5 5	32	3 5 5	45	4 5 5	81	5 5 5	2400+

* Number of positive tubes with each of 3 volumes used.

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CHAPTER XVII

DETECTION AND ENUMERATION OF FOOD-POISONING TYPES OF MICROORGANISMS

The methods that have been recommended in other chapters have been concerned with isolation and identification of microorganisms normal to food products or those that are an index of sanitation or cause spoilage and deterioration. These methods have been for informational, survey, or quality control purposes. In order not to cause confusion and not to repeat methods common to many types of food the material on the isolation of food-poisoning types of bacteria have been set aside in this separate chapter for use when the occasion dictates.

Food-poisoning outbreaks are encountered only upon occasion and result principally from accident, carelessness, and mishandling of food products in such a way as to allow the development of food-poisoning organisms to dangerous proportions. However, on occasion a situation presents itself in which it is desirable, or even essential, that information be obtained as to the presence or absence of organisms that may be potential health hazards, or to insure that such living organisms are not present in a particular lot of food.

In certain cases it is essential that assurance be given that the processing employed on the food is adequate to prevent the growth of anaerobic spore formers or that the pasteurization employed is sufficient to inactivate potentially dangerous nonspore-forming organisms. Also, it is often essential that the laboratory establish safety on foods of unknown history, origin, processing, or handling. In other cases the laboratory is required to examine food products believed to be involved in a food-poisoning outbreak. It is to fill these needs that the following methods are presented for the detection and enumeration of types of organisms known to be the cause of food-borne infections or intoxications.

If the laboratory worker is faced with a clinical situation involving a food-poisoning outbreak where the examination involves material other than food, or involves the use of animals, he should turn to

"Diagnostic Procedures and Reagents" [1] for methods and guidance and rely on material presented here only for those methods concerned with food. Also, he should realize that under these conditions his findings furnish only collaborative evidence to the investigation on the outbreak and that it is often necessary to piece together all phases of the problem before a solution can be found. Under such circumstances the worker may find it necessary to present his findings factually and not attempt an interpretation of what they mean to the case.

This chapter is intended only for use in the examination of food and only methods for the more common organisms that grow in food and cause poisoning through food are presented. Certain of the following procedures will be found in "Diagnostic Procedures and Reagents," [1] but are given here in order to present a more complete volume on food examination.

SAMPLING AND SAMPLE PREPARATION

The sampling procedure presented in each of the preceding chapters will in most cases be adequate for purposes of this chapter. Any deviations will be presented under each special heading.

1. In sampling take every precaution to prevent contamination of the product with extraneous organisms. The laboratory worker should accept foods that have been previously opened with reluctance and suspicion. In the laboratory one is able to sterilize container surfaces and aseptically open food products with assurance, but no such assurance can be given to products opened by untrained personnel outside the laboratory.

2. When bulk products or opened containers are presented for examination a complete report of the circumstances, condition of product, and anything unusual should be made a matter of record. The worker will often have no information as to the chance for contamination, temperature of holding, or time that the sample may have undergone incubation, etc. Under such circumstances it behooves him to make a record of all available information concerning the particular sample and to submit his findings based upon the record of the sample as received. Record all code marks and special information regarding the sample.

3. Protect the sample material from conditions known to activate growth or to kill particular organisms under study.

4. Examine carefully the condition of original opened, or unopened container. Record any signs of abnormality, such as swelling, perforations, or defective closures.

5. Examine and record conditions of the food product, noting especially signs of spoilage as denoted by cloudy liquid, gas evolution, or abnormal odor. *Do not taste products suspected as causes of food poisoning.*

6. Carry out examination for food-poisoning organisms promptly in order that bacteriological conditions at the time can be recorded and not after a possible shift in the flora has taken place.

7. Record and report fully the conditions, media, incubation temperatures, etc., used in the investigation in order that others may draw conclusions from the report presented.

8. Mix, grind, or blend sample material in such a manner as to obtain homogeneous material for inoculation into differential media or for the making of serial dilutions.

MICROSCOPIC EXAMINATION

If there is reason to suspect that a food has caused a food-poisoning outbreak, or is heavily contaminated, make smears directly from the sample or from low serial dilutions. This may provide information as to what to expect in making other types of examination. Such examination should be carried out even though the food may have undergone a heating process, for even though the product is found sterile the presence of large numbers of organisms such as Gram-positive cocci may indicate trouble since *Staphylococcus* enterotoxin is not destroyed by heating sufficiently to kill the organisms.

Prepare smears on slides, fix with heat, and stain with Gram stain (B3). Slides of fatty material should be treated with xylol before staining. Observe under the microscope for predominant types of organism with special reference to clostridia, Gram-positive cocci, and Gram-negative bacilli.

In addition, such an examination may be a valuable guide as evidence of improper sanitation or temperature of holding the food.

CLOSTRIDIUM BOTULINUM

I. Determination of Viable *Cl. botulinum*

A. *Enrichment Technic*

In cases where information on the presence of viable *Cl. botulinum* vegetative cells or spores is desired, or where toxin has not been demonstrated in a suspected food, use the following enrichment technic:

1. Inoculate 6 large tubes of freshly heated and cooled beef heart medium (7) or ground meat medium (43) with 1 gm or ml of the food sample.

2. Heat 4 tubes in a water bath at 80° C for 20 min and cool. This will inactivate vegetative cells and reduce contamination, but if only vegetative cells are present the unheated tubes will so indicate.

3. Incubate all tubes anaerobically for 4 days at 37° C. If an anaerobic jar is not available, layer each tube with sterile petrolatum or with thioglycolate agar (60).

4. After incubation note evidence of bacterial growth and make a Gram stain (B3) of material from the tubes. Examine microscopically for presence of Gram-positive bacilli with terminal or subterminal spores (clostridia).

5. Select tubes showing evidence of presence of clostridia and centrifuge to remove the solid material and bacteria. Test for presence of toxin by methods given under III, A or B, or in "Diagnostic Procedures and Reagents." [1]

B. *Direct Culture Technic*

If there is evidence that anaerobic spoilage has taken place, *Cl. botulinum* may be present in the food in large numbers and the food may be inoculated into the medium without dilution.

Streak a loopful of fluid from the sample in succession on 2 agar plates using blood agar (9) or meat infusion agar (41).

Incubate under anaerobic conditions at 37° C for 2–3 days. Examine for colonies of anaerobes.

Pick suspected colonies to beef heart medium (7), which have been heated and cooled before inoculation, and incubate anaerobically at 37° C for 4 days. Make Gram stain as under A.

Centrifuge tubes and use supernatant fluid for feeding or injection tests for toxin according to III, A or B or "Diagnostic Procedures and Reagents." [1]

II. Enumeration of Viable Anaerobic Spore Formers

It is often desirable to learn the extent of contamination with clostridia. The following procedures are recommended for obtaining an estimate of their numbers.

A. *Plate Technic*

Prepare serial dilutions of the food and heat for 20 min at 80° C. Transfer 1 ml to Petri plates, in duplicate, and pour with Andersen's medium (4),[2] mix thoroughly and allow to solidify.

Overpour with a heavy layer of thioglycolate agar (60) and incubate under anaerobic conditions at 32° C for 5–6 days.

Count anaerobic colonies and report as numbers of anaerobic spore formers.

B. *Tube Technic*

An alternate method to A may be employed making use of Veillon culture tubes.[3]

Plug the bottom of a 200-mm x 15-mm tube with a No. 0 rubber stopper and the top with a cotton plug. Fill with Andersen's medium (4) leaving a 2-in. head space and autoclave for 30 min at 121° C (15 lb pressure).

Inoculate tubes of medium, melted and cooled to about 45° C, with 2 ml of appropriate dilutions and mix thoroughly but carefully so as not to incorporate air. Solidify and layer with a 1-in. layer of thioglycolate agar (60).

Incubate at 32° C for 5–6 days and count dilutions showing distinct colonies. Report as numbers of colonies of anaerobic spore formers.

Isolations can be made by pushing the agar from the tubes and fishing material from isolated colonies. Also, microscopic examination to confirm the presence of clostridia may be made from such colonies.

If isolations are not to be made and only counts are desired an oval tube with a round neck [4] should be used. This tube can be counted conveniently on a colony counter.

III. Determination of *Cl. botulinum* Toxin in Food

Where the safety of a food is in doubt or where anaerobic spoilage is evident the following procedures are recommended for determination of the presence of toxin:

Prepare a water or saline extract from the food and centrifuge to remove solid material and bacterial cells. If a sterile filtrate is desired Seitz or Berkefeld filtration may be used.

Heat a portion of the liquid in a test tube for 10 min in a boiling water bath to serve as a heat-labile toxin control.

A. *Feeding Technic*

Feed guinea pigs with from 2- to 5-ml portions of unheated material and a similar group with like portions of the heated control. While this is not a delicate test animals receiving unheated material will show typical symptoms in the presence of toxin of high titer.

B. *Injection Technic*

Inject at least 2 mice intraperitoneally with from 0.1 to 0.5 ml of the unheated liquid and a similar number of mice with the same amount of heated control.

If the animals receiving the heated control survive while those receiving unheated control material die, the presence of botulinum toxin may be indicated. If none of the animals die, proceed with similar studies using enrichment cultures developed under I, A or B. If both sets die the presence of a heat-stable toxic material is indicated.

Upon occasion nonspecific deaths occur in mice injected with low dilutions due to certain toxic components in the food sample. By making injections with higher dilutions this effect can be eliminated.

If guinea pigs are used instead of mice, inject 0.5–2.0 ml of the test material intraperitoneally.

If there is need for further identification of the specific type of toxin or the use of protective antitoxin in the above tests, consult "Diagnostic

Procedures" [1] for details and procedures, or cultures can be sent to a laboratory equipped to conduct specific serological identification tests.

Animals receiving toxin of high potency are likely to die within a few hr after showing bellows-like respiration, constricted abdomen, and limb paralysis. Animals with lesser doses may live for 3 or 4 days exhibiting flaccidity of abdominal muscles and a dragging of the limbs. When using food material for injection purposes death may occur from bacterial infection and not toxin. This adds to the importance of using heated control material.

STAPHYLOCOCCUS

Certain of the staphylococci are responsible for food-poisoning outbreaks. Tanner [5] devotes 46 pages to discussion of these organisms in this role. The ability to produce enterotoxin is apparently limited to those strains that are coagulase-positive,[6, 7] however, not all coagulase-positive strains produce enterotoxin. As pointed out by Chapman, Lieb, and Curcio [8] many typical food-poisoning staphylococci usually produce pigment, coagulate plasma, hemolyze rabbit blood, ferment mannitol, and are gelatinase-positive. The staphylococci are not inhibited by 7.5 per cent sodium chloride,[9] and advantage is taken of this to facilitate isolation in the presence of other bacterial types. These facts should be borne in mind by the laboratory worker in making reports of findings on this type of organism. No single reaction can be depended upon to identify potential enterotoxin formers. Identification by cultural characteristics of potentially pathogenic staphylococci does not take the place of feeding or injection tests where positive demonstration of food-poisoning capability is needed. The full history of the samples and collaborative information should be taken into consideration.

ISOLATION AND IDENTIFICATION

Prepare a homogeneous sample of food material by making a 1:10 dilution in a sterile blender cup or use serial dilutions prepared according to directions in the various other chapters of this volume. Inoculate the following:

A. *Blood Agar (9)*

Streak sample material, or the various dilutions, on the surface of blood agar (9) plates for determination of hemolytic types of organisms.

Incubate at 32° C for 3 days and count typical staphylococci colonies showing hemolysis. A beta-type hemolysis will be found on blood agar plates for most strains of potential food-poisoning strains. Select isolated colonies for Gram-stain, coagulase, and other tests as indicated.

B. *Mannitol Salt Agar* (40)

The growth of organisms other than staphylococci is greatly restricted on this medium. Typical coagulase-positive colonies often show a yellow zone while coagulase-negative types are surrounded by red or purple zones.

1. Inoculate the surface of mannitol salt agar plates (40) with 0.1 ml of each of the decimal dilutions of food and spread evenly over the surface with a sterile bent glass rod.

2. Incubate at 32° C for 3 days and count typical yellow colonies. Report as numbers of suspect staphylococci.

3. Select isolated colonies and inoculate brain heart infusion broth (10) and incubate at 32° C for the coagulase test under E.

C. *Staphylococcus Medium No. 110* (57)

This medium has the advantage of showing pigmentation, mannitol fermentation, and gelatinase formation on a single medium.

1. Inoculate the surface of the medium with 0.1 ml of each decimal dilution and spread evenly over the surface with a sterile bent glass rod.

2. Incubate at 32° C for 3 days and count the typical *Staphylococcus* pigmented colonies. In most cases coagulase-positive staphylococci produce pigmentation on this medium while the negative types produce white colonies; however, *S. albus* strains may produce enterotoxin and should not be ignored.

3. After picking typical colonies for the coagulase and other tests place a drop of bromcresol purple indicator (C5) on several typical colonies. Any change in color from that of the background indicates mannitol fermentation.

4. Flood the plate with 5 ml of a saturated solution of ammonium sulfate or with a 20 per cent solution of sulfosalicylic acid which has been kept in the incubator with the plates for temperature control. Clear

zones around colonies or around where colonies were picked indicates a positive gelatinase reaction.

5. If desired, colonies picked under 3, above, may be streaked on blood agar (9) plates or inoculated into brain-heart infusion broth (10) for other tests.

D. *Tellurite Glycine Agar (61)*[10]

1. Pour 20 ml of tellurite glycine agar in sterile Petri plates and allow to solidify. Dry the surface of the agar by incubating overnight in an inverted position.

2. Transfer 0.1 ml aliquot of appropriate dilutions of sample to the center of the Petri plate and spread evenly over the surface with a sterile bent glass rod.

3. Incubate at 37° C for 24 hr. Coagulase-positive staphylococci produce black colonies. Longer incubation is not advised as coagulase-negative colonies will appear and produce black colonies.

E. *Coagulase Test*

Use material from a 24-hr culture in brain heart infusion broth (10) for this test.

1. Tube Technic

Dissolve 1 ampule of coagulase plasma in 3 ml of distilled water. Place 0.5 ml of plasma solution in a Wassermann tube.

Add 2 drops of 24-hr culture of suspected *Staphylococcus* grown in brain heart infusion broth (10) and place in incubator at 37° C.

Take a reading in 1 hr when clotting of the plasma is regarded as "coagulase-positive." Some cultures coagulate slower and readings should be made up to 3 hr when all tubes not coagulated may be considered negative.

2. Slide Technic

The slide technic can be used directly with colonies fished from plates.

Place a drop of water on a microscope slide and into it mix material from a suspected colony with a minimum of spreading. At the same

time on another slide make a Gram stain (B3) and observe for Gram-positive cocci.

Mix a large loop of coagulase plasma solution with the suspension of organisms on the slide and observe for clumping by gently rotating the slide before a light.

Coagulase-positive staphylococci will produce visible clumping within a few seconds. Cultures that clump very slowly or not at all should be regarded as coagulase-negative.

INTERPRETATION OF RESULTS

Gram-positive staphylococci isolated from food that are coagulase-positive should be regarded as potential food-poisoning types. Coagulase production is probably the only single test which if negative excludes possibility of enterotoxin production. Other tests are not as clearly diagnostic.

The finding of a small number of staphylococci in a food is not necessarily significant even if some of them are coagulase-positive. However, the finding of large numbers should cast suspicion on the handling, sanitation, and temperature of holding of the product. These organisms do not grow well at temperatures below 50° F and their presence in large numbers in a chilled or frozen product is an indication of previous improper handling.

Any food product having upward of half a million coagulase-positive staphylococci per gm should be considered a public health hazard and a careful check made of all phases of manufacture or handling of the product.

It must be remembered that the enterotoxin of this type of organism is not inactivated by heat and a product that has been heated to a point where all staphylococci are killed may still be dangerous. In such cases a microscopic examination showing large numbers of Gram-positive cocci should alert the worker to possible danger and indicate that feeding and injection tests should be made.

If there is need for the demonstration of enterotoxin in food or in culture filtrates by feeding or injection methods refer to "Diagnostic Procedures and Reagents." [1]

STREPTOCOCCUS

Streptococci have been implicated in food poisonings on infrequent occasions,[11, 12] and were shown by Dack, et al.,[13] to cause illness when fed to human volunteers in pure culture. Specifically the organism implicated in most cases has been *Streptococcus faecalis*.

In food suspected of causing food poisoning or checked as a quality control measure the presence of large numbers of streptococci should be viewed with concern, except in those foods where an acid fermentation has taken place as a part of the preparation, such as fermented milks, cheese, certain sausages, etc. Good judgment must be used in interpretation of results in light of the food product being examined.

No physiological or serological differences have been detected between streptococci implicated in food-poisoning cases and similar organisms isolated from normal sources. Also, since human feeding is the only known means of demonstrating the food-poisoning capabilities of streptococci, no definite conclusion can be drawn on the potentialities of food poisoning by the mere isolation and identification of such organisms as *S. faecalis*. Thus, the finding of abnormal numbers of enterococci in a food, in which few if any streptococci were to be expected, casts suspicion on the food and indicates that steps should be taken to improve the handling and processing sanitation and the control over temperature of holding of the food.

If the sample is a clinical one or examination is required beyond the scope of the methods presented here, refer to "Diagnostic Procedures and Reagents."[1] If specific identification of isolates is required, carry out differential tests suggested by Bergey's "Manual of Determinative Bacteriology."[14]

PREPARATION OF SAMPLE

Prepare sample as given in other chapters for microbiological examination or emulsify 1 part of food in 4 parts of sterile water. From this the serial dilutions may be made or media inoculated directly.

MICROBIOLOGICAL EXAMINATION

Prepare serial dilutions from above emulsion guided by the observations made during the microscopic examination as to relative numbers of organisms.

I. Liquid Culture

Where only determination of the presence of enterococci, or where an enrichment period is desired, inoculate serial dilutions into buffered azide glucose glycerol broth (14). Incubate for 48 hr at 37° C.

Acid production in this medium is strong evidence of the presence of enterococci. Color change from purple to yellow is generally made in 24 hr.

Where further assurance of presence of enterococci is desired transfer loops full of the incubated broth to tubes of tryptone glucose extract salt broth (69) and incubate at 37° C for 48 hr. Turbidity and the presence of Gram-positive streptococci in this high salt medium are very good evidence that the organisms are enterococci. As an alternate medium ethyl violet azide broth (26) may be used.

II. Agar Culture

A. *Enumeration*

Transfer 1 ml of appropriate dilutions of sample to Petri plates, in duplicate, and pour with enterococcus agar (25).[13]

Incubate at 37° C for 48 hr. Most colonies found on the plates will be enterococci, although an occasional *Lactobacillus* colony may be observed.

B. *Confirmation*

Make Gram stain (B3) of colony and observe for Gram-positive streptococci.

Fish colony to tryptone glucose extract salt broth (69) and incubate at 37° C for 48 hr. Observe for turbidity and presence of Gram-positive streptococci.

Fish colony to dextrose broth (18). Seal surface of broth with paraffin petroleum jelly or use method of Williams and Campbell [15] for detecting CO_2 production. Incubate at 45° C for 48 hr and observe for gas formation. Growth at 45° C with failure to produce visible quantities of carbon dioxide from dextrose is further confirmation for presence of enterococci.

For more extensive methods of identification of isolates see Sherman.[16] For serological grouping consult "Diagnostic Procedures and Reagents." [1]

SALMONELLA

Under normal circumstances food products as eaten do not contain bacteria belonging to this genus. When bacteria of the genus *Salmonella* are found in food products they are generally considered as constituting a potential public health hazard. Certain strains of this genus are considered more dangerous than others since they have been incriminated more frequently in food-poisoning outbreaks.

Tanner [5] and Dack [17] devote considerable space to the discussion of food-poisoning cases reported as being due to members of this genus. Edwards, et al.,[18] recorded wide distribution of these organisms in the United States and the sources of isolation of various *Salmonella* types. They showed that the types most frequently isolated from outbreaks were, in order of their frequency: *S. typhimurium, S. newport, S. choleraesuis, S. oranienburg, S. paratyphi* B, *S. montevideo,* and *S. panama.*

In view of the above it is essential that methods be available for the detection and enumeration of members of the genus *Salmonella* when occasion arises, such as for quality control, determining efficiency of sanitary and pasteurization procedures, and the determination of the cause of certain types of food poisoning.

SAMPLING AND PREPARATION OF SAMPLE

When samples are taken for quality control or for routine checking purposes they should be taken at such points and at such intervals as to be representative of the lots being produced or checked. Under these circumstances sampling procedure and sample preparation set forth in the various chapters are to be used.

MICROBIOLOGICAL EXAMINATION

From the vast amount of investigation in this field it is concluded that the use of selective enrichment media is to be recommended over attempts at direct isolation. However, if the population of Gram-negative short rods is very high direct methods may be attempted in the interest of conserving time, but not to the exclusion of enrichment procedures. With dried eggs and other dehydrated foods Byrne, et al.,[19] and Kunz and Ouchterlony [20] have shown preenrichment to be beneficial

for optimum recovery of *Salmonella*. This may be accomplished by incubating a suspension of the product in a suitable nutrient for 24 hr prior to transfer to selenite broth or other selective enrichment media.

I. Direct Inoculation Method

a) Streak a loopful of emulsified food material or a loopful from a low serial dilution on the surface of 2 bismuth sulfite agar (8) Petri plates consecutively. Repeat on the surface of 2 SS agar plates (56). This should be done in such a manner as to obtain well isolated colonies at some point along the streak on the 2 plates. The agar surface should be dried by leaving the Petri plate lids ajar after the pouring to allow escape of moisture while the agar is solidifying.

b) Incubate SS agar plates for 24 hr at 37° C and bismuth sulfite agar plates 48 hr. Observe for colonies showing *Salmonella* characteristics.

c) Fish suspected colonies to differential media for confirmation as set forth under III, Confirmatory Methods.

II. Enrichment Method

A. *Detection and Isolation*

1. Weigh 20 gm of food material or emulsified material into 80 ml of steamed selenite broth (54).* Mix thoroughly. If the food does not readily disperse add a spoonful of sterile glass beads and shake until good dispersion is obtained. As an alternate tetrathionate broth (62) may be used. This medium is not as convenient since the iodine solution must be added as the medium is used.

2. Incubate for 16–24 hr at 37° C.

3. Streak a loopful of the broth on the "dry" surface of bismuth sulfite agar (8) and on SS agar(56) and/or brilliant green agar (11).

4. Incubate the SS agar plates for 24 hr at 37° C and the bismuth sulfite plates for 48 hr. Observe for isolated colonies having *Salmonella* characteristics.

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* The addition of 10 mg per liter of cystine to selinite broth has been shown by Byrne, et al.,[19] and by North [21] to increase recovery of *Salmonella*.

It is recommended that in cases where the numbers of *Salmonella* are low, and typical colonies are not obtained on the initial streaking, the enrichment material be streaked on the surface of differential agars on 3 consecutive days in an attempt to make isolations.

5. For presumptive identification fish isolated suspected colonies from each plate and inoculate SIM medium (sulfide-indole-motility medium) (55) and make slant and stab inoculations in triple-sugar iron agar (65).

6. Incubate for 24 hr at 37° C and observe for typical *Salmonella* reactions as indicated under III. Colonies that are suspected of being *Salmonella* on bismuth sulfite agar, SS agar or brilliant green agar, show indole-negative in SIM medium and acid butt and no change on the slant of triple sugar iron agar may be presumptively considered *Salmonella.*

7. For further identification use purified cultures and inoculate differential media as directed under III, Confirmatory Methods.

B. *Enumeration by Most Probable Number (MPN) Technic*

1. Weigh 11 gm of sample into a 99-ml water blank (23F) containing glass beads to make 1:10 dilution or 1 gm per each 10 ml of solution. Shake thoroughly on a shaking machine for 30 min to obtain a uniform suspension.

2. Prepare appropriate decimal dilutions by pipetting 11 ml of each dilution into a 99-ml water blank (23A).

3. Pipette 10-ml aliquots into 5 replicate tubes containing 10 ml of double-strength selenite broth (54) for each of the serial dilutions and shake thoroughly. This will give an equivalent of 1.0, 0.1, 0.01, 0.001, and 0.0001 gm of food material per tube, respectively.

4. Incubate for 24 hr at 37° C.

5. Streak a large loopful of broth on the dry surface of freshly prepared bismuth sulfite agar (8) plates, SS agar plates (56), and/or brilliant green agar (11) in such a way as to obtain well isolated colonies.

If large numbers of samples must be run a plate may be divided into 4 segments. Streak each segment with a separate sample, using a small loop and first puncturing the agar at the outside edge before beginning

the streak. Care should be taken not to create aerosols which may contaminate neighboring segments.

6. Incubate SS agar plates for 24 hr at 37° C and the bismuth sulfite agar plates for 48 hr and observe for colonies with *Salmonella* characteristics.

7. For presumptive identification fish isolated suspected colonies from each plate and inoculate SIM medium (55) and make slant and stab inoculations in triple sugar iron agar (65).

8. Incubate for 24 hr at 37° C and observe for typical *Salmonella* reactions as indicated under III.

9. Record the number of tubes considered positive for *Salmonella* in each dilution. Select the 3 highest positive dilutions and determine the numerical value by consulting Table 1, Chapter XVI.

10. Calculate the MPN (Most Probable Number) according to the following:

$$\frac{\text{Number from MPN table}}{100} \times \text{dilution of middle tube} = \text{MPN per gm of sample.}$$

It will be noted that the MPN table is based on 100 gm (ml) of sample. As example of calculations, assume the following:

Dilution	1:10	1:100	1:1,000	1:10,000
Series A—Positive tubes	5	5	3	1
Series B—Positive tubes	4	3	1	0

For Series A the number corresponding to 5, 3, 1 is 110. Therefore,

$$\frac{110}{100} \times 1{,}000 = 1{,}100 \textit{ Salmonella} \text{ (MPN) per gm of sample.}$$

For Series B the number corresponding to 4, 3, 1 is 33. Therefore,

$$\frac{33}{100} \times 100 = 33 \textit{ Salmonella} \text{ (MPN) per gm of sample.}$$

In cases where "skips" occur considerable judgment must be exercised in choosing the dilutions to use. At times the different types of differential media will indicate different numbers. This is generally due to the presence of particular strains of *Salmonella* and the dilutions found positive may vary with the streaking medium. For example, Byrne, et al.,[19] have shown that the frequency of isolation of *S. pullorum*

is greater on bismuth sulfite agar than on SS agar or brilliant green agar.

However, as a rule one or more of the plate series will give clear-cut results and thus simplify the choosing of the dilutions.

III. Confirmatory Methods

It is not possible to definitely identify an organism as *Salmonella* on the basis of its characteristics on a single differential medium. It is, therefore, necessary to resort to the use of several types of media designed to confirm certain characteristics associated with the genus *Salmonella*. If identification as to exact type of *Salmonella* is necessary resort to serological typing and biochemical reactions.

Cultures obtained from bismuth sulfite agar, SS agar, and brilliant-green agar plates should be purified by plating or streaking on tryptone agar (66) before being inoculated into confirmatory media. Direct inoculation from a colony should be used only as a preliminary screening method with positive identification being made from purified cultures.

1. Triple Sugar Iron Agar

Inoculate purified culture into triple sugar iron agar (65) slants with deep butts, making both a streak and a stab inoculation. Incubate for 24 hr at 37° C. Make observations on the slant and the butt. Acid formation is indicated by a yellow color. *Salmonella* are characterized by no production of acid on the slant and with acid or acid and gas in the butt. H_2S formation may be $\pm$ with most cultures showing a positive reaction.

2. Sulfide Indole Motility Medium

Inoculate SIM medium (55) tubes using the stab method. Incubate 24 hr at 37° C.

a) Motility

Observe for motility along the stab. Motile cultures produce a "brush" or turbid appearance for a considerable distance from inoculation line.

b) Hydrogen Sulfide Production

Observe for blackening along inoculation line as a positive indication of hydrogen sulfide production.

c) Indole Formation

At the time of inoculation insert an oxalic acid paper indicator strip (C7) into the neck of the tube by placing on the side of the cotton plug allowing at least ½ in. of the paper to extend into the tube past the cotton plug. Take care that the paper does not become moistened or touch medium. After incubation observe strip for a pink color indicating a positive indole reaction. In the SIM medium reactions for *Salmonella* are Indole —; Motility ±, most strains being motile; H_2S formation ±, with most strains being positive.

Indole may also be determined by culturing in tryptone broth (67) and using Kovac's reagent (C1) for indole.

3. Urease Test

Make heavy inoculations of purified cultures into urea broth (70) for rapid differentiation between *Proteus* and *Salmonella*. Incubate at 37° C for 48 hr. Make observations at desired intervals depending upon urgency of identifying cultures. Any change in color from yellow to red indicates a positive urease reaction typical of the *Proteus* group. With heavy inoculations positive reactions can often be observed in a few hours.

Rapid Urease Reagent—

Inoculate heavily a tube containing 0.1–0.5 ml of the rapid urease reagent (C3) with the test organism from an agar slant or from a triple sugar iron agar slant.

Place at 37° C, preferably in a water bath, and observe for evidence of decomposition of the urea, indicated by marked alkalinization (red coloration) of the medium.

Record reaction after 15–30 min and at 1 hr. With heavy inoculations most urease-producing bacteria will develop the characteristic reaction within 30 min.

4. Serological Typing*

In those laboratories where *Salmonella* differential sera are available they may be used for identification as to particular groups. However,

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* The only presently known commercial source of polyvalent and group sera is Lederle Laboratories Division, American Cyanamid Company, New York 20, N. Y.

cost and scarcity of such material makes it difficult for the average laboratory to go this far in *Salmonella* identification. Therefore, if serological typing cannot be carried out by the laboratory worker, and there is need for such differentiation, he should send culture to a laboratory for typing where such sera are available.*

a) Slide Technic

This technic has the advantages of being very rapid and requiring a very small amount of serum.

Place a small drop of polyvalent *Salmonella* serum on a clean, dry microscope slide or ruled glass plate and with a straight inoculation wire transfer a small amount of purified culture from the agar slant to the drop. Quickly mix to a uniform suspension using the wire as a stirring device.

Grasp the slide between the thumb and forefinger and with a gentle figure 8 motion mix the material in the drop, taking care not to allow liquid to run across slide. By holding above an illuminated surface or at an angle against a light source observe condition of the suspension. If after thorough mixing there is evidence of clumping or a "grainy" appearance the serological reaction is positive. The slide may be observed under the low power of the microscope for evidence of clumping although this is ordinarily not necessary.

Where many plates must be examined or a screening method is desirable isolated colonies may be fished directly from the plate to the slide. Such procedure should be considered only as a screening method and not depended upon for positive identification. Several typings can be carried out simultaneously on the same slide by marking off small sections with a wax pencil.

If the reaction is positive, repeat above procedure using group sera for those groupings contained in the polyvalent serum. Evidence of clumping or graininess with a certain serum indicates positive identification for that group. Whenever a nonspecific reaction is obtained a saline control agglutination should be run as spontaneous agglutination is found in certain cultures.

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* Most state public health laboratories do such typing.

The following types are to be found in the various groupings:

Group	Salmonella Types Most Frequently Isolated
A	*S. paratyphi* A
B	*S. paratyphi* B, *S. typhimurium, S. derby, S. bredeny*
C_1	*S. oranienburg, S. montevideo, S. tennessee, S. bareilly, S. cholerae-suis*
C_2	*S. newport, S. kentucky, S. muenchen* (oregon), *S. manhattan*
D	*S. typhi, S. enteriditis, S. pullorum, S. gallinarum, S. panama, S. javiana*
E	*S. anatum, S. meleagridis, S. senftenberg, S. give, S. newington*
F, G, H, I, and further groups	*S. worthington, S. cerro, S. minnesota, S. urbana*

Since several *Salmonella* types may be found within the group it is necessary to proceed further by serological and biochemical means to make identification as to specific type. For such typing the laboratory worker should refer to Edwards and Ewing, "Identification of Enterobacteriaceae." [22]

b) Tube Technic

Place a small amount of polyvalent serum in a small serum tube. Transfer a small amount of culture from the slant to the tube by means of a straight inoculation needle.

Mix the solution thoroughly to obtain a uniform suspension of organisms and observe for clumping by holding before a light or by observing with a hand lens. A coagulated or grainy appearance as contrasted to a uniform cloudiness is evidence of a positive serological reaction.

If a positive reaction is obtained repeat the procedure using each group serum indicated in the polyvalent type until a positive reaction is obtained for a specific *Salmonella* grouping. If it is necessary to go further in typing proceed as indicated in "Identification of Enterobacteriaceae." [22]

If a nonspecific agglutination is obtained run a saline control to eliminate the possibility of a spontaneous agglutination reaction.

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22. Edwards, P. R., and Ewing, W. H. Identification of Enterobacteriaceae. Minneapolis, Minn.: Burgess Publishing, 1955.

APPENDIX A—CULTURE MEDIA

This appendix contains the formulas for all the culture media mentioned in the text of the various chapters, and the directions for making and using the media. The names of the various media are for the most part those by which they are known in the literature.

In the text the recommended medium is referred to by name and then in parentheses the number corresponding to the medium number of this appendix is given: thus "dextrose broth (18)." The prefix A was dropped since most references in the text are for media and in the interest of economy of space and printing only the number is used for this section.

In most cases media recommended here can be obtained in dehydrated form. However, if one wishes to make the media from the raw materials this section provides full information. Also, it was believed that formulation information would be of interest to those engaged in research. Only in rare instances is the name of any company given and then only to provide a source for material that is available from or is manufactured exclusively by a single company.

1. Acetate Lactate Medium [1]

Sodium acetate	5	gm	Soluble starch	1	gm
Calcium lactate	5	"	Sodium thioglycolate	0.5	"
Proteose peptone	5	"	Agar	5	"
Tryptone	5	"			

Dissolve ingredients by bringing to a boil. Adjust the pH to 6.5. Dispense into tubes and sterilize for 15 min at 121° C (15 lb pressure).

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1. Kawatomari, T., and Vaughn, R. H. Species of *Clostridium* Associated with Zapatera Spoilage of Olives. Food Research 21, 4:481, 1956.

2. Acetate Lactate Agar

Prepare as for Acetate-Lactate Medium (1) except that 20 gm of agar are used and the agar is dispensed in flasks or bottles for use as a pouring medium.

3. Amos and Kent-Jones Medium [1]

Peptone	10	gm	Sodium chloride	9	gm
Beef extract	5.4	"	Distilled water	1	liter

Dissolve ingredients by heating to boiling. Adjust to pH 7.2. Dispense in 5 ml amounts into tubes and sterilize by autoclaving for 15 min at 121° C (15 lb pressure).

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1. Amos, A. J., and Kent-Jones, D. W. The "Rope" Spore Count of Flour and Its Significance. Analyst 56:572–586, 1931.

4. Andersen's Medium [1]

Pork infusion*	800	ml	Dipotassium phosphate	1.25	gm
Pea infusion†	200	"	Soluble starch	1	"
Peptone	5	gm	Sodium thioglycolate	0.5	"
Tryptone	1.6	"	Agar	16	"

Mix ingredients and adjust to pH 7.2. Autoclave mixture for 5 min at 121° C (15 lb pressure) and clarify while hot by adding 25 gm of "Celite" and filtering through Whatman No. 4 filter paper with suction. Tube in 14 ml quantities and store in closed container in freezer. As needed autoclave tubes for 12 min at 121° C (15 lb pressure).

Prepare solution of 5 gm of $NaHCO_3$ to 100 ml of water and sterilize by Seitz filtration. Keep in refrigerator.

When pouring plates add 0.4 ml of the $NaHCO_3$ solution to each plate, or 1 ml to each 14 ml tube of medium. Mix thoroughly with the inoculum and allow to solidify.

Overpour with thioglycolate agar (60).

If tubes are used for culturing pour 1 in. layer of thioglycolate agar over solidified agar column.

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1. Andersen, A. A. A Rapid Plate Method of Counting Spores of *Clostridium botulinum*. J. Bact. 62:425–432, 1951.

* Pork infusion. Add 1 lb of fresh lean ground pork to 1 liter of water and steam for 1 hr. Filter out the meat through cheese cloth. Solidify the fat by chilling and remove. Centrifuge to remove remaining solids and use liquid in the formula.

† Pea infusion. Blend 1 lb of fresh, good quality, green peas or frozen peas with 1 lb of water and steam for 1 hr. Remove solids by centrifuging and clarify by filtration with "Celite" as under pork infusion. Use filtrate in the formula.

5. A.P.T. Agar [1] *

Tryptone	12.5	gm	Tween 80	0.2	gm
Yeast extract	7.5	"	Magnesium sulfate	0.8	"
Monopotassium phosphate	5	"	Manganous chloride	0.14	"
Sodium chloride	5	"	Ferrous sulfate	0.04	"
Sodium citrate	5	"	Agar	15.0	"
Sodium carbonate	1.25	"	Distilled water	1	liter
Dextrose	10	"			

Dissolve ingredients and dispense into flasks or bottles. Sterilize for 12 min at 121° C (15 lb pressure). Longer sterilization is not recommended. The final pH should be approximately 6.7.

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1. Evans, J. B., and Niven, C. F., Jr. Nutrition of the Heterofermentative Lactobacilli that Cause Greening of Cured Meat Products. J. Bact. 62:599–603, 1951.

* This medium may be used as a broth by withholding the agar.

6. Azide Dextrose Broth [1]

Beef extract	4.5	gm	Sodium chloride	7.5	gm
Tryptone or polypeptone	15.0	"	Sodium azide	0.2	"
Dextrose	7.5	"	Distilled water	1	liter

Dissolve ingredients and dispense into tubes in 10 ml amounts. Sterilize at 121° C (15 lb pressure) for 15 min. The final reaction should be pH 7.2.

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1. Mallmann, W. L., and Seligmann, E. B., Jr. A Comparative Study of Media for the Detection of Streptococci in Water and Sewage. A.J.P.H. 40:286, 1950.

7. Beef Heart Infusion Medium [1]

Trim the fat from a beef heart (or if not available beef tissue) and grind or mince 500 gm of material. Add 1,000 ml of tap water and allow to stand overnight in a refrigerator.

Remove from refrigerator and boil over a free flame for 15 min or steam in an Arnold steam chest for 30 min.

Separate the tissue from the liquid by passing through 2 layers of cheese cloth and save both portions.

To the liquid portion add 10 gm peptone and 5 gm of NaCl and restore to original volume.

Adjust to pH 7.6 with 1 *N* NaOH and boil for 15–20 min or heat in an Arnold steam chest for 30 min, and filter through paper.

If medium is to be used immediately:
Place a 2-cm column of tissue in a test tube and fill the tube half full with the broth. Sterilize for 45 min at 121° C (15 lb pressure).

If the medium is to be stored:
Place the liquid in screw-capped bottles and sterilize for 15 min at 121° C (15 lb pressure). Rapidly dry the tissue in a thin layer in an incubator with forced circulation and store in a sealed jar. When medium is needed prepare tubes as above and sterilize as for the freshly made tubes.

USE: Check tubes for sterility by incubation for 24 hr at 37° C. This medium may be used for the cultivation of pathogenic and other anaerobes and is suitable for carrying stock cultures and demonstrating spore formation. It is not suitable for the butyric-butyl group of thermophilic anaerobes.

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1. Manual of Methods for Pure Culture Study of Bacteria (9th ed.). Geneva, N. Y.: Biotech. Pub., 1944, II 44, p. 16.

8. Bismuth Sulfite Agar

Beef extract	5	gm	Bismuth sulfite indicator	8	gm
Peptone	10	"	Brilliant green	0.025	"
Dextrose	5	"	Agar	20	"
Disodium phosphate	4	"	Distilled water	1	liter
Ferrous sulfate	0.3	"			

Add ingredients to water and bring to a boil. Frequent agitation is necessary to prevent burning on bottom. Cool, slightly agitate, and pour a thick layer into Petri plates, making sure that flocculant precipitate is evenly distributed. Set cover aside so that about 1/3 of plate is exposed for escape of moisture vapor and to dry agar surface. A dry surface will produce distinct individual colonies along streak. Final pH should be approximately 7.7. Do not sterilize this medium in autoclave or by fractional sterilization in Arnold steamer.

9. Blood Agar

Infusion from beef heart*	500	gm	Agar	15	gm
Tryptose or Thiotone	10	"	Distilled water	1	liter
Sodium chloride	5	"			

Add ingredients to water and bring to a boil. Dispense in tubes or flasks and autoclave for 15 min at 121° C (15 lb pressure). Final reaction should be approximately 6.8.

When pouring blood agar plates melt medium and cool to 45°–50° C and add aseptically 5 per cent of sterile defibrinated blood. Mix thoroughly avoiding incorporation of air bubbles and distribute in sterile tubes or pour into Petri plates in a thick layer.

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* Prepare as described in Meat Infusion Broth (42).

10. Brain Heart Infusion Broth

Calf brain, infusion from*	200	gm	Dextrose	2	gm
Beef heart, infusion from*	250	"	Sodium chloride	5	"
Peptone	10	"	Disodium phosphate	2.5	"

Dissolve ingredients in the water by bringing to a boil and make up to 1 liter. Dispense into tubes and autoclave for 15 min at 121° C (15 lb pressure). Final reaction should be approximately pH 7.4.

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* Infuse with equal weight of water as described under Meat Infusion Broth (42).

11. Brilliant Green Agar [1]

Peptone	10	gm	Phenol red	0.08	gm
Sodium chloride	5	"	Brilliant green	0.0125	"
Yeast extract	3	"	Agar	20	"
Lactose	10	"	Distilled water	1	liter
Saccharose	10	"			

Add ingredients to the water and heat to boiling. Dispense in tubes or flasks and autoclave for 15 min at 121° C (15 lb pressure). Do not oversterilize and cool at once. The final reaction should be approximately pH 6.9.

When used for streaking, melt agar in boiling water and pour into Petri plates in a thick layer. Set cover aside so that about 1/3 of plate is exposed for escape of moisture vapor and to dry agar surface. A dry surface will produce distinct individual colonies along streak.

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12. Brilliant Green Lactose Bile Agar [1]

Peptone	8.25	gm	Erioglaucine	0.0649	gm
Lactose	1.9	"	Basic fuchsin	0.0776	"
Oxgall	0.00295	"	Brilliant green	0.0000295	"
Sodium sulfite	0.205	"	Agar	10.5	"
Ferric chloride	0.0295	"	Distilled water	1	liter
Monopotassium phosphate	0.0153	"			

Dissolve ingredients by bringing to a boil. Dispense into tubes or flasks and autoclave for 15 min at 121° C (15 lb pressure). Final reaction should be approximately pH 6.9. The medium is sensitive to light and should be stored in the dark.

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1. Noble, R. E., and Tonney, F. O. Solid Brilliant Green Lactose Bile Medium for Direct Plating With Results in Seventeen Hours. J. A. Water Works A. 27:108, 1935.

13. Brilliant Green Lactose Bile Broth, 2 Per cent [1]

Peptone	10	gm	Brilliant green	0.0133	gm
Lactose	10	"	Distilled water	1	liter
Oxgall	20	"			

Dissolve the peptone and lactose in approximately 500 ml of distilled water.

Add 20 gm of dehydrated oxgall dissolved in 200 ml of water. The pH of this solution should be between 7.0 and 7.5.

Mix the 2 solutions and make up to approximately 975 ml. Adjust reaction to pH 7.4.

Add 13.3 ml of a 0.1 per cent aqueous brilliant green in distilled water.

Add sufficient distilled water to make 1 liter, dispense into fermentation tubes and autoclave for 15 min at 121° C (15 lb pressure).

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1. Standard Methods for the Examination of Water, Sewage, and Industrial Wastes (10th ed.). New York: American Public Health Association, 1955.

14. Buffered Azide Glucose Glycerol Broth [1]

Tryptose or polypeptone.....	20	gm	Sodium chloride	5	gm
Glucose	5	"	Sodium azide	0.5	"
Dipotassium phosphate	4	"	Glycerol	5	ml
Monopotassium phosphate ...	1.5	"	Distilled water	1	liter

Add the glycerol to the distilled water and then dissolve other ingredients by gentle heating. Dispense into tubes in 8–10 ml amounts and sterilize by autoclaving for 15 min at 117° C (10 lb pressure). Do not use 121° C. Final reaction should be approximately pH 6.9.

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1. Hajna, A. A. Pub. Health Lab. 9:80, 1951.

15. Buffered Boric Acid Lactose Broth [1]

Proteose peptone or polypeptone	10	gm	Monopotassium phosphate (anhydrous)	4.085	gm
Lactose	5	"	Boric acid	3.25	"
Dipotassium phosphate (anhydrous)	12.203	"	Distilled water	1	liter

Dissolve ingredients and dispense in 20 ml amounts into fermentation tubes. Sterilize for 15 min at 121° C (15 lb pressure) and cool immediately after withdrawal from the autoclave. Final reaction should be pH 7.0.

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1. Vaughn, R. H.; Levine, M.; and Smith, H. A. A Buffered Boric Acid Lactose Medium for Enrichment and Presumptive Identification of *Eschrichia coli.* Food Research 16, 1: 10–19, 1951.

16. Desoxycholate Lactose Agar

Peptone	10	gm	Sodium desoxycholate	0.5	gm
Lactose	10	"	Neutral red	0.03	"
Sodium chloride	5	"	Agar	15	"
Sodium citrate	2	"	Distilled water	1	liter

Dissolve by bringing to a boil and adjust to pH 7.1. There is no need to further sterilize unless medium is to be stored. Overheating will inhibit solidification of agar. Use as a pouring medium for coliform bacteria.

17. Dextrose Agar. Acidified

Beef extract	3	gm	Sodium chloride	5	gm
Tryptose or polypeptone	10	"	Agar	15	"
Dextrose	10	"	Distilled water	1	liter

Add ingredients to water and bring to a boil. Dispense into tubes or flasks and autoclave for 15 min at 121° C (15 lb pressure). When

used plating medium for yeasts and molds, melt agar in flowing steam, cool to approximately 50° C. Add sufficient sterile 10 per cent tartaric acid solution to adjust to approximately pH 3.5.

18. Dextrose Broth

Beef extract	3 gm	Sodium chloride	5 gm
Tryptose or trypticase	10 "	Agar	1 "
Dextrose	5 "	Distilled water	1 liter

Dissolve ingredients by heating and dispense in tubes in 8–10 ml amounts. Sterilize by autoclaving for 15 min at 121° C (15 lb pressure).

19. Dextrose Broth Plus Salt [1]

Beef extract	3 gm	Dextrose	20 gm
Tryptose or trypticase	10 "	Distilled water	1 liter

Dissolve by bringing to a boil.

1. Low Salt—Add 50 gm of sodium chloride
2. High Salt—Add 100 gm of sodium chloride

Dissolve the salt and then dispense into tubes. Autoclave for 15 min at 121° C (15 lb pressure).

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1. Etchells, J. L., and Jones, I. D. Procedure for Bacteriological Examination of Brined, Salted, and Pickled Vegetables and Vegetable Products. A.J.P.H. 36:1121 (Oct.), 1946.

20. Dextrose Tryptone Agar [1]

Tryptone or trypticase	10 gm	Agar	15 gm
Dextrose	5 "	Distilled water	1 liter

Heat to boiling to dissolve ingredients. Dispense into tubes or flasks and autoclave for 15 min at 121° C (15 lb pressure). Final reaction should be approximately pH 6.8.

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1. Standard Methods for the Examination of Dairy Products (10 ed.). New York: American Public Health Association, 1953, p. 185.

21. Dextrose Tryptone Bromcresol Purple Agar

Prepare as above in (20). Add 0.04 gm of bromcresol purple as an indicator.

22. Dextrose Tryptone Bromcresol Purple Broth

Tryptone or trypticase	10	gm	Bromcresol purple	0.04	gm
Dextrose	5	"	Distilled water		liter

Adjust to approximately pH 6.8, tube in 8–10 ml a , and sterilize for 15 min at 121° F (15 lb pressure).

23. Dilution Blanks

A. *Dilution Blanks, Plain*

Use 6-oz bottles of resistant glass closed with rubber stoppers or screw caps. Use nontoxic tap water or buffered distilled water. Do not use waters of abnormally high mineral content. Measure water accurately into bottles so they contain 99 ml after sterilization. Autoclave for 20 min at 121° C (15 lb pressure).

B. *Dilution Blanks with Sand*

Prepare as in A and add approximately 10 gm of purified sea sand to each bottle before sterilization. Autoclave for 20 min at 121° C (15 lb pressure).

C. *Lithium Hydroxide Dilution Blanks*

Prepare as under A and add 4.19 gm of crystalline lithium hydroxide to each 1,000 ml of water. Autoclave 20 min at 121° C (15 lb pressure).

D. *Phosphate Buffered Dilution Blanks*

Stock Solution

Potassium acid phosphate (KH_2PO_4)	34 gm
Distilled water	500 ml

Adjust to pH 7.2 with *N* sodium hydroxide and make up to 1 liter with distilled water.

Dilution Blanks

Take 1.25 ml of above stock solution and make up to 1 liter with distilled water. Dispense into bottles to produce 99-ml portions and sterilize for 20 min at 121° C (15 lb pressure).

E. *Saline Dilution Blanks*

Prepare as in A and add 8.5 gm of sodium chloride to each 1,000 ml of water.

F. *Saline Dilution Blanks with Glass Beads*

Prepare as in E and add a tablespoonful of Pyrex glass beads before sterilization. Dry glass beads may be sterilized in flasks and added to saline dilution blanks (E) as needed.

24. Endo Agar

Peptone	10.0 gm	Basic fuchsin	0.4 gm
Lactose	10.0 "	Agar	15.0 "
Dipotassium phosphate	3.5 "	Distilled water	1 liter
Sodium sulfite	2.5 "		

Dissolve ingredients by heating to boiling and distribute into tubes or flasks. Sterilize for 15 min at 121° C (15 lb pressure). The final pH should be approximately 7.5. If the medium is to be used the same day as made up it may be poured into Petri plates without autoclaving. Before pouring gently agitate to disperse the precipitate.

25. Enterococcus Agar [1, 2]

Tryptone or trypticase	5 gm	Agar	15 gm
Glucose	5 "	Sodium azide	0.1 "
Yeast extract	5 "	Penicillin	100 Oxford units
		Distilled water	1 liter

Dissolve ingredients by heating, with the exception of the penicillin, and dispense in measured amounts into bottles or flasks. Sterilize by autoclaving for 15 min at 121° C (15 lb pressure).

When ready for plating cool agar to approximately 45° C and add the proportional amount of penicillin. Mix thoroughly and pour into Petri plates.

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1. Dack, G. M.; Niven, C. F., Jr.; Kirsner, J. B.; and Marshall, H. Feeding Tests on Human Volunteers with *Enterococci* and Tyramine. J. Infect. Dis. 85:131–138, 1949.
2. White, J. C., and Sherman, J. M. Occurrence of Enterococci in Milk. J. Bact. 48:262, 1944.

26. Ethyl Violet Azide Broth [1]

Tryptose or biosate	20 gm	Sodium chloride	5 gm
Dextrose	5 "	Sodium azide	0.4 "
Dipotassium phosphate	2.7 "	Ethyl violet	0.00083 "
Monopotassium phospate	2.7 "	Distilled water	1 liter

Dissolve ingredients and distribute in tubes in 10 ml amounts. Sterilize at 121° C (15 lb pressure) for 15 min. The final reaction should be pH 7.0.

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1. Litsky, W.; Mallmann, W. L.; and Fifield, C. W. Comparison of the Most Probable Numbers of *Escherichia coli* and Enterococci in River Waters. A.J.P.H. 45, 8:1049 (Aug.), 1955.

27. Holman's Alkaline Cooked Meat Medium [1]

Infuse 500 gm of ground fresh lean beef in 1 liter of water overnight in a refrigerator. Skim off fat. Strain infusion through several layers of cheese cloth and press out broth. Retain the pressed beef cake. Make broth to original volume with water and add 5 gm of peptone. Heat in steam chest for 10 min. Filter and add 5 gm of sodium chloride. Add normal sodium hydroxide until alkaline to phenolphthalein. Heat in steam chest for 15 min and filter. Transfer about 2 gm of pressed out beef to each test tube (150 x 20 mm), add 10 ml of clear alkaline broth to tubes, and autoclave at 121° C for 20 min. Final pH should be 7.2–7.4. Store media at 40°–45° F. Immediately before use, heat tubed medium in boiling water for at least 15 min to expel adsorbed oxygen and cool promptly to 37° C before inoculation.

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1. Official Methods of Analysis of the Association of Official Agricultural Chemists (7th ed.), 1953.

28. Jensen's Pork Sucrose Nitrate Medium [1]

Fill culture tubes approximately ½ full with finely ground or chopped spiced ham (from a sound can) and wet down with a solution composed of 0.4 per cent sodium nitrate and 2 per cent sucrose. The meat should be covered at least ¼ in. by solution. No trapped bubbles of air should appear in medium. Tubes should not be more than ½ full of meat and broth. Autoclave for 15 min at 121° C (15 lb pressure).

When used inoculate at the bottom of medium, taking care not to introduce air bubbles.

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1. Jensen, L. B.; Wood, I. H.; and Jansen, C. B. Swelling in Canned Chopped Hams. Ind. Eng. Chem. 26:1118, 1934.

29. Lactose Broth [1]

Beef extract	3 gm	Lactose	5 gm
Peptone	5 "	Distilled water	1 liter

Dissolve ingredients in water by heating to 65° C and dispense in fermentation tubes. Autoclave for 15 min at 121° C (15 lb pressure). Final reaction should be approximately pH 6.9.

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1. Standard Methods for the Examination of Water, Sewage, and Industrial Wastes (10th ed.). New York: American Public Health Association, 1955.

30. Lauryl Tryptose Broth [1]

Tryptose	20.0 gm	Sodium chloride	5.0 gm
Lactose	5.0 "	Sodium lauryl sulfate,	
Dipotassium phosphate	2.75 "	Duponol (W A Flakes) *	0.1 "
Monopotassium phosphate	2.75 "	Distilled water	1 liter

Dissolve ingredients and distribute into fermentation tubes. Sterilize for 15 min at 121° C (15 lb pressure). Final pH should be approximately 6.8.

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1. Standard Methods for the Examination of Water, Sewage, and Industrial Wastes (10th ed.). New York: American Public Health Association, 1955.

* Manufactured by E. I. duPont de Nemours & Company, Wilmington, Del.

31. Levine EMB Agar [1, 2]

Peptone	10 gm	Agar	15 gm
Lactose	10 "	Eosin Y	0.4 "
Dipotassium phosphate (K_2HPO_4)	2 "	Methylene blue	0.065 "
		Distilled water	1 liter

Add ingredients to water and bring to a boil. Dispense into tubes or flasks and sterilize by autoclaving for 15 min at 121° C (15 lb pressure). Final reaction should be approximately pH 7.1.

Avoid overheating as agar is likely to become soft and unsuitable for streaking. When used as a streaking medium melt agar in flowing steam or boiling water. Gently swirl contents of flask to distribute flocculant precipitate and pour thick layer into Petri plates. Set cover aside so as to leave about ⅓ of plate exposed for escape of moisture vapor and to dry surface of agar after solidification. A dry surface promotes growth of distinct individual colonies and better differentia-

tion as to type. Plates should be used at once but may be stored for a short period inverted in the refrigerator. When stored check for contamination before use.

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1. Levine, M. Iowa Engineering Experiment Station Bull. No. 62, 1921.
2. Also see Standard Methods for the Examination of Water, Sewage, and Industrial Wastes (10th ed.). New York: American Public Health Association, 1955.

32 Liver Broth Medium [1]

Add 500 gm of finely ground beef liver to 1,000 ml of distilled water and boil slowly 1 hr. Adjust to pH 7.0 and boil for another 10 min. Press through cheese cloth to remove liver particles and make liquid up to 1,000 ml with water. Add 10 gm of peptone and 1 gm of dipotassium phosphate, and adjust reaction to pH 7.0.

Place about ½ in. of liver particles from the pressed cake in bottom of tubes (⅝ x 6″) and cover with broth. Autoclave for 20 min at 121° C (15 lb pressure). Before use exhaust for 20 min in flowing steam.

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1. Cameron, E. J. Report on Culture Media for Non-Acid Products. J. A. Off. Agr. Chem. 19:433, 1936.

33. Liver Broth Plus Salt [1]

Prepare as above in (32).

Add 15 per cent sodium chloride, by weight, to the broth and add to liver particles in the tubes. Sterilize as above.

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1. Etchells, J. L., and Jones, I. D. Procedure for Bacteriological Examination of Brined, Salted and Pickled Vegetables and Vegetable Products. A.J.P.H. 36:1121 (Oct.), 1946.

34. Liver Broth with Crushed Tomatoes

This medium is useful for the isolation of butyric acid forming anaerobes and other acid-tolerant bacteria from spoiled cans of food or from cannery line samples, because it inhibits the growth of various interfering species.

Prepare the liver broth as presented in (32).

Liver broth	500 ml
Crushed tomatoes	500 "

Adjust pH to 5.0 and tube medium over liver particles, as stated in (32). Sterilize for 20 min at 117° C (10 lb pressure).

The addition of 10 per cent soil renders medium more favorable for spore formation. When soil is added sterilize for 20 min at 121° C (15 lb pressure)

35. Liver Infusion—Sorbic Acid Agar [1]

Make liver broth as indicated in liver broth medium (32). To 1 liter of liver broth add:

Sorbic Acid	120 gm
Tryptone or trypticase	10 "
Agar	20 "

Dissolve ingredients by bringing to a boil. Adjust pH to approximately 5.5. Sterilize for 15 min at 121° C (15 lb pressure).

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1. Emard, L. O., and Vaughn, R. H. Selectivity of Sorbic Acid Media for the Catalase Negative Lactic Acid Bacteria and Clostridia. J. Bact. 63:487, 1952.

36. Malt Agar

Malt extract powder	30 gm
Agar	15 "
Distilled water	1 liter

Dissolve ingredients by heating to boiling. Dispense in tubes or flasks and autoclave for 15 min at 121° C (15 lb pressure). When used as plating medium for yeasts and molds melt in flowing steam or boiling water and acidify to pH 3.5 with sterile lactic acid solution. To preserve solidifying properties of the agar do not heat the medium after the addition of the acid.

37. Malt Dextrose Agar [1]

Prepare as in (36) above.

Add 150 gm of dextrose if used for French dressing or add 300 gm of dextrose if used for mayonnaise.

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1. Fabian, F. W., and Wethington, M. C. Spoilage in Salad and French Dressings Due to Yeasts. Food Research 15:134, 1950.

38. Malt Extract Agar, Trommer's [1]

Malt extract (Trommer's)	30 gm
Agar	20 "
Distilled water	1 liter

If used for French dressing add 150 gm dextrose.

If used for mayonnaise add 300 gm dextrose. Dispense into tubes or flasks and autoclave 15 min at 121° C (15 lb pressure).

1. Fabian, F. W., and Wethington, M. C. Bacterial and Chemical Analyses of Mayonnaise, Salad Dressings and Related Products. Food Research 15:138, 1950.

39. Malt Salt Agar [1]

1. Malt extract powder	20 gm	2. Sodium chloride	74 gm
Agar	20 "	Distilled water	300 ml
Distilled water	750 ml		

Sterilize each solution by autoclaving for 15 min at 121° C (15 lb pressure). Before pouring Petri plates mix the 2 solutions and cool to about 45° C before pouring. Final reaction should be approximately pH 5.0.

1. Christensen, C. M. Quantitative Determination of Molds in Flour. Cereal Chemistry 23:322–329, 1946.

40. Mannitol Salt Agar [1]

Beef extract	1	gm	d-Mannitol	10	gm
Proteose peptone or polypeptone	10	"	Agar	15	"
			Phenol red	0.025	"
Sodium chloride	75	"	Distilled water	1	liter

Mix ingredients in water and heat to boiling to dissolve. Dispense in tubes or flasks and autoclave 15 min at 121° C (15 lb pressure). Final reaction should be approximately pH 7.4. Do not overheat medium and cool as soon as taken from autoclave.

1. Chapman, G. H. The Significance of Sodium Chloride in Studies of Staphylococci. J. Bact. 50:201, 1945.

41. Meat Infusion Agar

To the Meat Infusion Broth (42) add 15 gm of agar and bring to boil to dissolve agar. Dispense in tubes or flasks and autoclave for 15 min at 121° C (15 lb pressure).

42. Meat Infusion Broth [1]

Lean beef muscle	500 gm	Sodium chloride	5 gm
Peptone	20 "	Distilled water	1 liter

Remove all fascia and fat from the muscle and grind in a food mill. Infuse ground meat overnight in water at 4°–6° C in an enameled or stainless steel container. Heat to 45° C and hold for 1 hr at that temperature. Boil for ½ hr and allow to cool without stirring. Skim off fat and lift out fine coagulum. Pour off supernatant fluid and transfer to a meat press. Express juice and combine juice with supernatant liquid. Filter through glass wool and make up to original volume.

Dissolve peptone and salt in liquid and adjust to pH 7.4. Boil for 20 min and pour into tall glass cylinder to settle. Decant clear supernatant. Dispense into tubes or flasks and autoclave for 15 min at 121° C (15 lb pressure).

1. Diagnostic Procedures and Regents (3rd ed.). New York: American Public Health Association, 1950.

43. Meat Medium, Ground

To the Meat Infusion Broth (42) add 1 per cent dextrose and adjust to pH 7.2–7.6. Place enough of the ground pressed meat in culture tubes to fill tubes about ½ full and add liquid to cover. Autoclave for 15 min at 121° C (15 lb pressure).

44. Methylene Blue Agar [1]

Glucose	1 gm	Methylene blue	0.01 gm
Agar	2 "	Distilled water	100 ml

Dissolve ingredients by heating. Add 1 ml of 0.1 *N* NaOH. Sterilize by autoclaving 15 min at 121° C (15 lb pressure).

Use this agar in its reduced form as a seal for culture tubes. Atmospheric oxygen will gradually cause the surface to turn blue and to

progress downward. As long as the bottom of the seal is colorless anaerobic conditions are being maintained.

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1. Hall, I. C. Chemical Criteria of Anaerobiosis with Special Reference to Methylene Blue. J. Bact. 6, 1:1–44, 1920.

45. Nutrient Agar

Beef extract	3 gm	Agar	15 gm
Peptone	5 "	Distilled water	1 liter

Heat to boiling to dissolve ingredients. Dispense into tubes or flasks and autoclave 15 min at 121° C (15 lb pressure). Final reaction should be approximately pH 6.8.

46. Nutritive Caseinate Agar [1, 2]

Isoelectric casein	3	gm	Bromcresol purple	0.04	gm
Peptonized milk, dry	7	"	Distilled water	1	liter
Agar	12	"			

Heat to boiling to dissolve ingredients and dispense in tubes or flasks. Autoclave 15 min at 121° C (15 lb pressure). Final reaction should be approximately pH 6.5.

For use as a differential counting medium see Chapter III.

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1. Black, L. A.; Prouty, C. C.; and Graham, R. A. The Effect of Pasteurization on the Bacterial Flora of Low Count Milk. J. Dairy Sc. 15:99, 1932.
2. Prouty, C. C. Further Studies of the Effect of Pasteurization on the Bacterial Flora of Low Count Milk. J. Dairy Sc. 17:115, 1934.

47. Orange Serum Agar [1]

This medium is excellent for cultivating acid-tolerant organisms, including *B. coagulans*, *B. thermoacidurans*, the lactobacilli and supports vigorous growth of butyric acid-forming anaerobes in shake tubes. However, it is not satisfactory for counting the butyric anaerobes due to the large amount of gas produced.

Prepare orange serum by heating 1 liter of freshly extracted orange juice to approximately 93° C (200° F). Add 30 gm of filter aid and mix thoroughly. Filter under suction through a Buchner funnel using

coarse filter paper precoated with filter aid. Discard the first few ml of filtered serum.

Tryptone or trypticase.....	10 gm	Agar	17 gm
Yeast extract	3 "	Orange serum	200 ml
Dextrose	4 "	Distilled water	800 "
Dipotassium phosphate	3 "		

Sterilize 15 min at 121° C (15 lb pressure). After sterilization, the pH should be about 5.5.

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1. Stevens, J. W. Preparation of Dehydrated Agar Media Containing Orange Juice Serum. Food Technol. 8:88–92, 1954.

48. Orange Serum Broth

Use the same formula as Orange Serum Agar (47) withholding the agar.

49. Plain Agar for Overpouring

Agar	20 gm
Distilled water	1 liter

Dissolve agar by bringing to a boil. Dispense into tubes or flasks and autoclave 15 min at 121° C (15 lb pressure). Use as a layering agent in tubes or as overpour in Petri plates.

50. Polypectate Gel Medium [1]

Sodium polypectate*	60	gm	Calgon (food grade)	2.5	gm
Peptone	5	"	Distilled water	1	liter
Beef extract	3	"			

Prepare in 500 ml quantities or according to capacity of the laboratory blender.

Add dry ingredients to 150 ml of water in a laboratory blender and start mixing. Add 350 ml of boiling water and blend until a smooth, creamy mixture is obtained, generally about 2 to 3 min.

Dispense medium into tubes or flasks and sterilize for 15 min at 121° C (15 lb pressure).

Upon removal from autoclave place tubes at a slant to cool and solidify.

This medium can be melted like agar but reautoclaving is not recommended as it causes frothing and boiling over.

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1. Vaughn, R. H.; Levinson, J. H.; Nagel, C. W.; and Krumperman, J. H. Sources and Types of Aerobic Microorganisms Associated with the Softening of Fermenting Cumbers. Food Research 19:495–502, 1954.

* This product can be obtained from Exchange Orange Products Co., 616 East Sunkist Ave., Ontario, Calif.

51. Potato Dextrose Agar (Acidified)

Infusion from white potatoes*	200 ml	Agar	15 ml
Dextrose	20 "	Distilled water	800 "

Heat mixture to boiling to dissolve the ingredients. Distribute into tubes or flasks and autoclave 15 min at 121° C (15 lb pressure). When used as plating medium for yeasts and molds melt in flowing steam or boiling water, cool and acidify to pH 3.5 with a sterile 10 per cent tartaric acid solution. Mix thoroughly and pour into plates. To preserve solidifying properties of the agar do not heat medium after the addition of tartaric acid.

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* Boil sliced potatoes in 500 ml of water for 15 min or until soft. Filter through cotton and use as part of water in formula.

52. Proteose Peptone Acid Agar[1]

This medium is used for the detection of tomato juice spoilage organisms of the *B. thermoacidurans* type.

Proteose peptone or polypeptone	5 gm	Dextrose	5 gm
Yeast extract	5 "	Dipotassium phosphate	4 "
		Distilled water	500 ml

Adjust pH to 5.0 with hydrochloric acid. Sterilize 30 min at 121° C (15 lb pressure). To use, add the above to an equal volume of sterile melted 4 per cent agar solution.

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1. Stern, R. M.; Hegerty, C. P.; and Williams, O. B. Detection of *Bacillus thermoacidurans* (Berry) in Tomato Juice and Successful Cultivation of the Organism in the Laboratory. Food Research 7:186–191, 1942.

53. Rogosa, Mitchell, and Wiseman Medium [1]

Trypticase	10	gm	Sorbitan monooleate	1	gm
Yeast extract	5	"	Sodium acetate hydrate	25	"
Monopotassium phosphate	6	"	Acetic acid	1.3	ml
Ammonium citrate	2	"	Salt solution	5	"*
Dextrose	20	"	Agar	15	gm

Final pH should be approximately 5.4. Sterilize 15 min at 121° C (15 lb pressure).

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1. Rogoso, M.; Mitchell, J. A.; and Wiseman, R. F. A Selective Medium for the Isolation and Enumeration of Oral and Fecal Lactobacilli. J. Bact. 62:132, 1951.

* Prepare solution consisting of:

$MgSO_4$, $7H_2O$	11.5	gm	$FeSO_4$, $7H_2O$	0.68	gm
$MnSO_4$, $4H_2O$	2.8	"	Distilled water	100	ml

54. Selenite Broth [1]

Tryptone or polypeptone	5	gm	Sodium acid selenite	4	gm
Lactose	4	"	Distilled water	1	liter
Disodium phosphate	10	"			

Suspend ingredients in water and heat to boiling. Dispense into tubes or flasks to a depth of at least 2 in. Sterilize in flowing steam for 30 min and cool. Excessive steaming is to be avoided. Do not autoclave. Final reaction should be approximately pH 7.0.

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1. Leifson, E. New Selenite Enrichment Media for the Isolation of Typhoid and Paratyphoid (Salmonella) Bacilli. Am. J. Hyg. 24:423, 1936.

55. SIM (Sulfide Indole Motility) Medium

Formulation A			*Formulation B*		
Peptone	30.0	gm	Trypticase	20.0	gm
Beef extract	3.0	"	Thiotone	6.1	"
Peptonized iron	0.2	"	Ferrous ammonium sulfite	0.2	"
Sodium thiosulfate	0.025	"	Sodium thiosulfate	0.2	"
Agar	3.0	"	Agar	3.5	"
Distilled water	1	liter	Distilled water	1	liter

Dissolve ingredients by bringing to a boil. Dispense into tubes to a depth of about 3 in. Sterilize 15 min at 121° C (15 lb pressure). To use inoculate by making a single stab in center of the soft agar, incubate and observe for motility of the culture along stab, indole by the use of an oxalic acid indicator paper (C7) and for sulfide formation by darkening at the bottom of the stab.

56. SS Agar

Beef extract	5	gm	Sodium thiosulfate	8.5	gm
Proteose peptone or polypeptone	5	"	Ferric citrate	1	"
Lactose	10	"	Brilliant green	0.00033	"
Bile salts	8.5	"	Neutral red	0.025	"
Sodium citrate	8.5	"	Agar	13.5	"
			Distilled water	1	liter

Dissolve ingr[illegible] by bringing to a boil. Final reaction should be approxima[illegible]..0.

Do not sterilize in the autoclave.

As soon as all ingredients are in solution cool until the flask can be handled and pour about 20 ml of medium into each Petri plate. The plates should be in a draftless area of low contamination. After pouring, partially remove the covers of the dishes to allow vapor to escape and dry surface of the agar for 2 hr. When used for streaking agar surface should appear dry in order to obtain well isolated colonies.

57. Staphylococcus Medium 110 [1, 2]

Tryptone or trypticase	10	gm	Sodium chloride	75	gm
Yeast extract	2.5	"	Dipotassium phosphate	5	"
Gelatin	30	"	Agar	15	"
d-Mannitol	10	"	Distilled water	1	liter
Lactose	2	"			

Dissolve ingredients by heating to boiling. Dispense into tubes or flasks and sterilize 15 min at 121° C (15 lb pressure). The final pH should be 7.0. When cool enough to handle, but while still hot, gently agitate to disperse precipitate and pour into Petri plates.

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1. Chapman, G. H. A Single Culture Medium for Selective Isolation of Plasma-Coagulating Staphylococci and for Improved Testing of Chromogenesis, Plasma-Coagulation, Mannitol Fermentation and the Stone Reaction. J. Bact. 51:409, 1946.
2. Chapman, G. H. A Simple Method for Making Multiple Tests of a Microorganism. J. Bact. 63:147, 1952.

58. Starr's Spirit Blue Agar [1]

Peptone	10 gm	Cottonseed oil emulsion*	25 ml
Yeast extract	5 "	Agar	30 gm
Spirit blue, 0.3 per cent alcoholic solution	50 ml	Distilled water	1 liter

Dissolve agar, peptone, and yeast extract in approximately 900 ml of water by steaming for several min. Add cottonseed oil emulsion and the previously filtered alcoholic solution of spirit blue. Make up with water to 1 liter and mix thoroughly. Dispense into tubes or flasks and autoclave for 15 min at 121° C (15 lb pressure).

Medium may be stored in the refrigerator for long periods. Poured Petri plates of agar are pale lavendar in color and of firm consistency. Colonies and lipolytic organisms develop a permanent deep blue color beneath and surrounding the colony. Since medium is not inhibitory a total count and a lipolytic count may be made on the same plate.

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1. Starr, M. F. Spirit Blue Agar: A Medium for the Detection of Lipolytic Microorganisms. Science 93:333, 1941.

* Cottonseed oil emulsion. Prepare emulsion by grinding thoroughly in a mortar or colloidal mill: 100 ml of *fresh* cottonseed oil, 10 gm of finely powdered gum arabic and 400 ml of warm distilled water. Thorough grinding will produce a smooth permanent emulsion in which most of the fat globules are less than 10 micra in diameter. Cottonseed oils of high acidity are not satisfactory for making this emulsion. Commercial cottonseed salad oils are satisfactory unless rancid.

59. Sulfite Agar [1-3]

For the detection of thermophilic anaerobes producing H_2S (*Clostridium nigrificans*).

Tryptone or trypticase.....	10 gm	Agar	20 gm
Sodium sulfite (anhydrous)...	1 "	Water	1 liter

Dispense into tubes in about 15 ml amounts and into each tube place an iron nail or a small clean strip of iron or base plate. No adjustment of reaction is necessary. Autoclave 20 min at 121° C (15 lb pressure). Tubes should be used within a week after making.

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1. Standard Methods for the Examination of Dairy Products (10th ed.). New York: American Public Health Association, 1953, p. 199, par. 6.58.
2. Official Methods of Analysis of the Association of Official Agricultural Chemists (7th ed.). Washington, D. C.: Association of Official Agricultural Chemists, 1950, p. 740, par. 36.14.
3. Clark, F. M., and Tanner, F. W. Thermophilic Canned Food Spoilage Organisms in Sugar and Starch. Food Research 2:27, 1937.

60. Thioglycolate Agar [1]

Agar	20	gm
Sodium thioglycolate	0.5	"
Distilled water	1	liter

Add ingredients to water and bring to a boil. Dispense into tubes or flasks and autoclave 15 min at 121° C (15 lb pressure). Medium is used for overpour to provide anaerobic conditions.

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1. Andersen, A. A. A Rapid Plate Method of Counting Spores of *Clostridium botulinum*. J. Bact. 62:425, 1951.

61. Tellurite Glycine Agar [1]

A. Tryptone or trypticase	1.0 gm	Lithium chloride	0.5 gm
Yeast extract	0.5 "	Glycine	1.0 "
Mannitol	0.5 "	Agar	2.0 "
Dipotassium phosphate	0.5 "	Distilled water	100 ml

Adjust to pH 7.2. Sterilize 15 min at 121° C (15 lb pressure). B. Prepare a 1 per cent aqueous solution of potassium tellurite. Sterilize 15 min at 121° C (15 lb pressure).

To prepare for plating add 2 ml of solution B to medium A to give 0.02 per cent of potassium tellurite. Medium A should be cooled to approximately 50° C and B added aseptically. Pour 20 ml into Petri plates and after solidifying invert and incubate overnight to dry surface of the agar.

---

1. Zebovitz, E.; Evans, J. B.; and Niven, C. F., Jr. Tellurite-Glycine Agar: A Selective Plating Medium for the Quantitative Detection of Coagulase-Positive Staphylococci. J. Bact. 70, 6:686–690, 1955.

62. Tetrathionate Broth

Proteose peptone or polypeptone	5 gm	Calcium carbonate	10 gm
Bile salts	1 "	Sodium thiosulfate	30 "
		Distilled water	1 liter

To use, heat to boiling and cool to below 45° C. Add 20 ml of iodine solution * per liter of the above. Mix thoroughly and dispense into sterile tubes or flasks. The complete medium should be used the day it is prepared. Do not heat after the iodine is added.

If it is desirable to maintain a stock solution the above basic medium

may be sterilized by autoclaving and stored. Add the iodine solution to the stock solution before using.

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* Preparation of Iodine Solution

Potassium iodide	5 gm
Iodine crystals	6 "
Distilled water	200 ml

Dissolve ingredients and store in the dark.

63. Tomato Juice Agar

Tomato juice	400 ml	Agar	11 gm
Peptone	10 gm	Distilled water	1 liter
Peptonized milk	10 "		

Heat to boiling to dissolve ingredients and dispense into tubes or flasks. Autoclave 15 min at 121° C (15 lb pressure). Final reaction should be approximately pH 6.1.

64. Total Plate Count Agar

Formula A—Tryptone Glucose Yeast Agar [1, 2]

Tryptone	5.0 gm	Agar	15.0 gm
Yeast extract	2.5 "	Distilled water	1 liter
Glucose	1.0 "		

Dissolve ingredients in water by bringing to boil. Dispense into tubes or flasks and autoclave 15 min at 121° C (15 lb pressure). Final reaction should be pH 7.0.

---

1. Standard Methods for the Examination of Dairy Products (10th ed.). New York: American Public Health Association, 1953, p. 99, par. 2.16a:
2. Buchbinder, L.; Boris, Y.; and Goldstein, L. Further Studies on New Milk-free Media for the Standard Plate Count of Dairy Products. A.J.P.H. 43:869, 1953.

Formula B—M-P H Medium [1]

Milk-protein hydrolysate	9 gm	Agar	15 gm
Glucose	1 "	Distilled water	1 liter

Dissolve ingredients by bringing to a boil. Dispense into tubes or flasks and autoclave 15 min at 121° C (15 lb pressure). The final pH should be 7.0.

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1. Standard Methods for the Examination of Dairy Products (10th ed.). New York: American Public Health Association, 1953, p. 99, par. 2.16b.

65. Triple Sugar Iron Agar

Beef extract	3	gm	Dextrose	1	gm
Yeast extract	3	"	Sodium chloride	5	"
Peptone, Proteose peptone } or polypeptone	15	"	Ferrous sulfate	0.2	"
			Sodium thiosulfate	0.3	"
	5	"	Phenol red	0.024	"
Lactose	10	"	Agar	12	"
Saccharose	10	"	Distilled water	1	liter

Add ingredients to water and bring to a boil. Distribute in tubes using enough medium to obtain a deep butt. Autoclave 15 min at 121° C (15 lb pressure). Remove from autoclave and slant to obtain a deep butt. Final reaction should be approximately pH 7.4.

66. Tryptone Agar

This medium is used for stratifying tryptone broth (67).

Tryptone or trypticase	5 gm
Agar	20 "
Distilled water	1 liter

Adjust reaction to approximately pH 6.8. Tube and sterilize 15 min at 121° C (15 lb pressure).

67. Tryptone Broth [1]

This medium is used for the detection of thermophilic anaerobes not producing H_2S (*Cl. thermosaccharolyticum*), putrefactive anaerobes and other mesophilic anaerobes.

Tryptone or trypticase	10	gm	Yeast extract	1	gm
Dextrose	5	"	Distilled water	1	liter
Dipotassium phosphate	1.25	"			

Adjust to approximately pH 6.8, tube in 8–10 ml amounts and sterilize 20 min at 121° C (15 lb pressure).

Unless freshly sterilized tubes should be exhausted in flowing steam for at least 20 min before using.

After inoculation stratify tubes with melted tryptone agar (66).

If medium is to be incubated at 55° C the stratifying layer should be solidified by cooling and then tubes preheated in a water bath to 55° C before being placed in the incubator.

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1. Cameron, E. J. Report on Microbiological Methods for Detecting and Estimating Thermophilic Bacteria in Sugar. J. A. Off. Agr. Chem. 33:744, 1950.

68. Tryptone Broth, for Indole [1]

Tryptone	10 gm
Distilled water	1 liter

Dissolve tryptone and dispense into test tubes in 5 ml portions. Sterilize by autoclaving 15 min at 121° C (15 lb pressure).

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1. Standard Methods for the Examination of Water, Sewage, and Industrial Wastes (10th ed.). New York: American Public Health Association, 1955, p. 391.

69. Tryptone Glucose Extract Salt Broth

Tryptone or trypticase	5 gm	Sodium chloride	6.5 gm
Glucose	1 "	Distilled water	1 liter
Yeast extract	2.5 "		

Dissolve ingredients and tube in 8 to 10 ml amounts. Sterilize by autoclaving 15 min at 121° C (15 lb pressure). Final reaction should be approximately pH 7.0.

70. Urea Broth [1, 2]

Yeast extract	0.1 gm	Urea	20.0 gm
Monopotassium phosphate	9.1 "	Phenol red	0.01 "
Disodium phosphate	9.5 "	Distilled water	1 liter

Dissolve ingredients in water, sterilize by filtration and dispense under aseptic conditions in 1 to 3 ml amounts into sterile tubes. Final reaction should be approximately pH 6.8. Do not autoclave or steam sterilize this medium.

---

1. Rustigian, R., and Stuart, C. A. Taxonomic Relationship in the Genus *Proteus*. Proc. Soc. Exper. Biol. & Med. 53:241, 1943.
2. Stuart, C. A.; Van Stratum, E.; and Rustigian, R. Further Studies on Urease Production by Proteus and Related Organisms. J. Bact. 49:437, 1945.

71. Vaspar

Paraffin (melted)	250 ml
Vaspar (melted)	250 "

Mix the 2 melted products thoroughly and use as an anaerobic sealing material.

72. V-8 Medium for Lactobacilli [1]

V-8 vegetable juice*	500	ml	Agar	15	gm
Tryptose or biosate	10	gm	Bromcresol green	0.1	"
Lactose	5	"	Distilled water—sufficient to make total to	1	liter
Beef extract	3	"			

To prepare—filter V-8 vegetable juice through 2 layers of cheese cloth. Mix ingredients and dissolve by heating. Adjust reaction to pH 5.7. Sterilize by autoclaving 15 min at 121° C (15 lb pressure).

Strong lactic acid-producing colonies appear dark green to jet black and have a bright yellow halo. Weaker acid producers are lighter green and with a less pronounced halo.

---

1. Fabian, F. W.; Fulde, R. C.; and Merrick, J. E. A New V-8 Medium for Determining Lactobacilli. Food Research 18, 3:280–289, 1953.

* V-8 is a trade name for a blend of 8 vegetable juices and may be procured in the retail food market.

73. Violet Red Bile Agar

Yeast extract	3.0	gm	Neutral red	0.03	gm
Peptone	7.0	"	Crystal violet	0.002	"
Bile salts	1.5	"	Agar	15.0	"
Lactose	10.0	"	Distilled water	1.0	liter
Sodium chloride	5.0	"			

Suspend material in water and allow to stand for a few min. Mix thoroughly and adjust to pH 7.4. Heat with agitation and boil for 2 min. Cool to about 42° C and use as a plating medium. After solidification add a cover layer of plain agar (49) or of above agar, to prevent surface growth and spreading of colonies.

Incubate plates 18–24 hr at 37° C. Count those colonies, 1–2 mm in diameter, having a purplish red color and surrounded with a reddish zone of precipitated bile. Plates with less than 150 colonies should be counted for best results.

APPENDIX B—STAINS

B 1. Erythrosin Stain

Stock Solution

Erythrosin B, certified	1 gm
Distilled water	100 ml

Dissolve dye and store in the refrigerator.

Buffer Solution

Buffer solution is made by dissolving equal parts of 0.2 *M*, Na2HPO4 and 0.2 *M*, NaH_2PO4 in distilled water.

To Use

Make a 1:5,000 concentration of the stain by diluting 1 ml of the stock solution with 50 ml of the buffer solution.

To 1 ml liquid sample add 1 ml of the 1:5,000 erythrosin solution in a small serum tube. Shake to obtain an even suspension of organisms and transfer a drop of solution to the hemacytometer with a 3-mm platinum loop.

B 2. Gentian Violet Stain

Gentian violet, crystalline	1 gm
Distilled water	100 ml

B 3. Gram's Stain;[1] Hucker Modification

1. Ammonium Oxalate Crystal Violet Solution

Crystal violet (85 per cent) dye content, certified	4 gm
Ethyl alcohol (95 per cent)	20 ml
Dissolve crystal violet in the alcohol	
Ammonium oxalate	0.8 gm
Distilled water	80 ml

Dissolve the ammonium oxalate in the water.

Dilute the crystal violet solution 1:10 with distilled water.

Mix 1 part of diluted crystal violet solution and 4 parts of the ammonium oxalate solution.

2. Gram's Iodine Solution

Iodine	1 gm
Potassium iodide	2 "
Distilled water	300 ml

Dissolve iodine and potassium iodide in the water. Prepare solution at least every 2 weeks.

3. Counterstain

Safranin (2.5 per cent solution in 95 per cent alcohol)	10 ml
Distilled water	100 "

4. Staining Procedure
   a. Stain 1 min, with the crystal violet solution
   b. Wash in water
   c. Apply iodine solution for 1 min.
   d. Wash in water
   e. Decolorize in 95 per cent alcohol for 30 sec with gentle agitation or until violet dye fails to appear in the alcohol.
   f. Apply counterstain for 10 sec.
   g. Wash in water and dry without blotting.

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1. Diagnostic Procedures and Reagents (3rd ed.). New York: American Public Health Association, 1950, p. 87.

B 4. Gram's Stain, Kopeloff and Beerman's Modification

1. 1 per cent aqueous methyl violet	30 ml
5 per cent sodium bicarbonate solution	8 "
2. Iodine	1 gm
Potassium iodide	2 "
5 per cent sodium bicarbonate solution	60 ml
Distilled water	240 "

Dissolve as in Gram's stain (B3) and add sodium bicarbonate solution.

3. Flood slide with methyl violet solution and allow to remain 5 min.
   Wash with water.

Flood with iodine solution and leave 2 min.

Drain and decolorize with acetone.

Decolorization is very rapid and must be carefully controlled.

Wash in water.

Counterstain for 30 sec to 1 min with safranin or basic fuchsin.

Wash, blot, and dry.

4. Counterstain

a. Safranin—saturated alcoholic solution of safranin	10 ml
Distilled water	90 "
b. Basic fuchsin—	
Basic fuchsin	0.05 gm
Distilled water	100 ml

B 5. Gray's Double Dye Stain [1]

1. 1 per cent aqueous methylene blue	50 ml
Methanol	50 "
Add dye to the methanol.	
2. 1 per cent aqueous basic fuchsin	25 "
Methanol	25 "
Add dye to the methanol.	

Before use mix the two solutions. Stain slide for a few sec with the 2-stain dye, dry and examine. Bacteria are stained blue and the background pink.

If the slide needs defatting use xylene and wash off with methyl alcohol before staining.

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1. Gray, P. H. H. Two-Stain Method for Direct Bacteria Count. J. Milk Technol. 6, 2:6, 1943.

B 6. Malachite Green Stain

Malachite green	5 gm
Distilled water	100 ml

B 7. Methylene Blue Stain

Methylene blue, crystalline	1 gm
Distilled water	100 ml

B 8. North's Aniline Oil—Methylene Blue Stain

To prepare stain, mix 3 ml of aniline oil and 10 ml of 95 per cent ethyl alcohol. Add 1.5 ml of concentrated HCl slowly with constant agitation. Slowly add 30 ml of a saturated alcoholic methylene blue solution and then add distilled water to make 100 ml. Filter and keep in tightly stoppered bottle.

B 9. Safranin Stain

Safranin	0.5 gm
Distilled water	100 ml

B 10. Wolford's Stain [1]

1. Basic fuchsin	1 gm
Ethyl alcohol, 95 per cent	100 ml
2. North's aniline oil methylene blue stain (B8).	

Make dilute stain for making direct microscopic counts by mixing 2 ml of solution A with 10 ml of solution B. Filter before use.

The dilute stain solution may be kept under refrigeration for 3 to 4 weeks, but should be discarded after that time.

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1. Wolford, E. R. A Direct Microscopic Method Modified for Estimation of Microorganisms in California Frozen Citrus Concentrate. A I C No. 365, Agr. Research Service, U. S. Dept. of Agriculture (Nov.), 1953.

APPENDIX C—REAGENTS AND INDICATORS

C 1. Kovac's Reagent for Indole

p-Dimethylaminobenzaldehyde	5 gm
Amyl alcohol	75 ml
Hydrochloric acid, concentrated	25 "

Dissolve p-dimethylaminobenzaldehyde in the amyl alcohol and then add the hydrochloric acid.

To test for indole add 0.2 to 0.3 ml of the reagent to 5 ml of a 24-hr culture of bacteria in tryptone broth (67). A dark red color in the surface layer constitutes a positive test for indole.

C 2. Nitrite Reagent

Sulfanilic Acid Reagent	
Sulfanilic acid	1 gm
Acetic acid, 5*N*	125 ml
A-Naphthylamine Reagent	
A-Naphthylamine	1 gm
Acetic acid, 5*N*	200 ml

To determine presence of nitrite burn on fermented sausages streak first 1 reagent and then the other across the surface by means of pipettes. In cases of nitrite burn an intense red color develops on the discolored area.

C 3. Rapid Urease Reagent [1, 2]

A. Stock Urea Solution

Urea	4 gm
Ethyl alcohol, 95 per cent	4 ml
Distilled water	8 "

Do not sterilize

Store in refrigerator. If urea precipitates out, warm in the hand or in warm water until it dissolves.

B. Stock Buffer Solution

Dipotassium phosphate	0.1 gm
Monopotassium phosphate	0.1 "
Sodium chloride	0.5 "
Phenol red (0.2 per cent)	1.0 ml
Distilled water	100 "

Sterilize this solution in the autoclave.

Procedure

Dilute 1 part of solution A with 19 parts of solution B. This constitutes the test solution.

The mixture *must not* be heated and need not be sterilized by filtration. A fresh mixture should be made from the stock solutions each time it is used.

A marked red coloration of the solution is a positive reaction for bacterial urease production.

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1. Adler, H. E.; Willers, E. H.; and Levine, M. Incidence of Salmonella in Apparently Healthy Dogs. J. Am. Vet. M. A. 118:300–304, 1951.
2. Anderson, T. A Modification of the Urease Test for Proteus. Science 101:470, 1945.

C 4. Silver Nitrate Reagent

Silver nitrate	29.063	gm
Distilled water	1,000	ml

This makes a 0.171*N* silver nitrate solution. Dissolve silver nitrate, place in a brown bottle, standardize and store in refrigerator.

When 1 ml of sample is titrated, each ml of silver nitrate solution used is equal to 1 gm of sodium chloride per 100 ml of sample. Use dichlorofluorescein indicator (C6).

C 5. Bromcresol Purple Indicator [1]

Carefully weigh 0.1 gm of bromcresol purple indicator and grind in a mortar with 3.7 ml of 0.05*N* NaOH.

When the solution is complete make up to 25 ml with distilled water. This constitutes the stock solution.

To make up the indicator solution dilute 1 ml of the stock solution with 9 ml of distilled water.

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1. Standard Methods for the Examination of Water, Sewage, and Industrial Wastes (10th ed.). New York: American Public Health Association, 1955.

C 6. Dichlorofluorescein Indicator

Dichlorofluorescein	0.5 gm
95 per cent ethyl alcohol	100 ml

Use 3–5 drops of indicator when determining sodium chloride by titrating with silver nitrate reagent (C4). The end point is a light salmon pink color.

C 7. Oxalic Acid Indicator for Indole [1]

Prepare a saturated solution of oxalic acid. Soak filter paper in this solution and hang up to dry.

Cut paper into strips approximately ½ in. wide and 2–3 in. long.

To use as an indicator for indole formation: After inoculation of SIM agar (55) insert an oxalic acid indicator strip into the neck of the tube, bending the end of the strip over the lip of the tube, and insert the cotton plug. Take care that the strip does not touch the medium or become moist. If the strip turns pink during incubation it indicates a positive reaction for indole.

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1. Holman, W. H., and Gonzales, F. L. A Test for Indole Based on the Oxalic Acid Reaction of Gnezda. J. Bact. 8:577, 1923.

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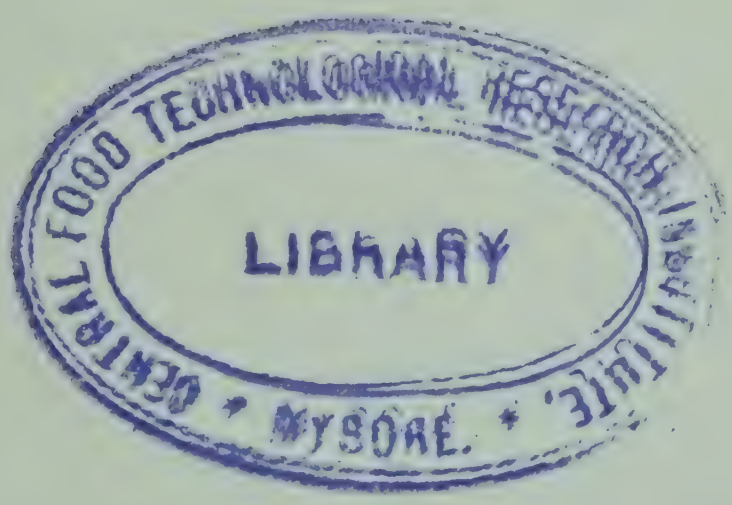

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